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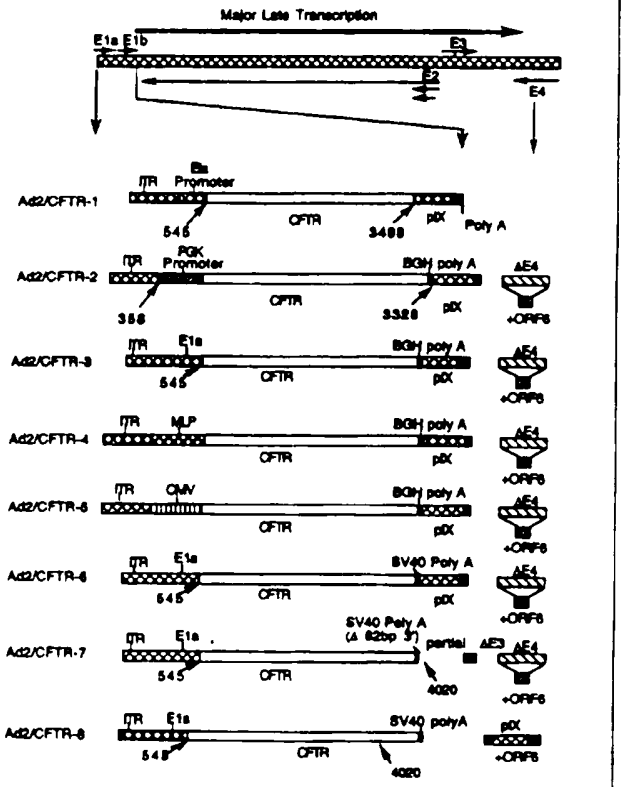
(57) Abstract

The present invention relates to novel adenovirus vectors for use in gene therapy which are designed to prevent the generation of replication-competent adenovirus (RCA) during *in vitro* propagation and clinical use. The invention also provides methods for the production of the novel virus vectors. These vectors maximize safety for clinical applications in which adenovirus vectors are used to transfer genes into recipient cells for gene therapy.



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Adenovirus vectors for gene therapy

Background of the Invention

The present invention relates to novel adenovirus vectors for use in gene therapy which are designed to prevent the generation of replication-competent adenovirus (RCA) during *in vitro* propagation and clinical use. The invention also provides methods for the production of the novel virus vectors. These vectors maximize safety for clinical applications in which adenovirus vectors are used to transfer genes into recipient cells for gene therapy.

Background Of The Invention

Prospects for gene therapy to correct genetic disease or to deliver therapeutic molecules depend on the development of gene transfer vehicles that can safely deliver exogenous nucleic acid to a recipient cell. To date, most efforts have focused on the use of virus-derived vectors that carry a heterologous gene (transgene) in order to exploit the natural ability of a virus to deliver genomic content to a target cell.

Most attempts to use viral vectors for gene therapy have relied on retrovirus vectors, chiefly because of their ability to integrate into the cellular genome. However, the disadvantages of retroviral vectors are becoming increasingly clear, including their tropism for dividing cells only, the possibility of insertional mutagenesis upon integration into the cell genome, decreased expression of the transgene over

time, rapid inactivation by serum complement, and the possibility of generation of replication-competent retroviruses (Jolly, D., *Cancer Gene Therapy* 1:51-64, 1994; Hodgson, C.P., *Bio Technology* 13:222-225, 1995).

Adenovirus is a nuclear DNA virus with a genome of about 36 kb, which has been well-characterized through studies in classical genetics and molecular biology (Horwitz, M.S., "Adenoviridae and Their Replication," in *Virology*, 2nd edition, Fields, B.N., et al., eds., Raven Press, New York, 1990). The genome is classified into early (known as E1-E4) and late (known as L1-L5) transcriptional units, referring to the generation of two temporal classes of viral proteins. The demarcation between these events is viral DNA replication.

Adenovirus-based vectors offer several unique advantages, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the potential to carry large inserts (Berkner, K.L., *Curr. Top. Micro. Immunol.* 158:39-66, 1992; Jolly, D., *Cancer Gene Therapy* 1:51-64, 1994). The cloning capacity of an adenovirus vector is about 8 kb, resulting from the deletion of certain regions of the virus genome dispensable for virus growth, e.g., E3, deletions of regions whose function is restored in trans from a packaging cell line, e.g., E1, and its complementation by 293 cells (Graham, F.L., *J. Gen. Virol.* 36:59-72, 1977), as well as the upper limit for optimal packaging which is about 105%-108% of wild-type length.

Genes that have been expressed to date by adenoviral vectors include p53 (Wills et al., *Human Gene Therapy* 5:1079-188, 1994); dystrophin (Vincent et al., *Nature Genetics* 5:130-134, 1993; erythropoietin (Descamps et al., *Human Gene Therapy* 5:979-985, 1994;

ornithine transcarbamylase (Stratford-Perricaudet et al., *Human Gene Therapy* 1:241-256, 1990); adenosine deaminase (Mitani et al., *Human Gene Therapy* 5:941-948, 1994); interleukin-2 (Haddada et al., *Human Gene Therapy* 4:703-711, 1993); and α1-antitrypsin (Jaffe et al., *Nature Genetics* 1:372-378, 1992).

The tropism of adenoviruses for cells of the respiratory tract has particular relevance to the use of adenovirus in gene therapy for cystic fibrosis (CF), which is the most common autosomal recessive disease in Caucasians, that causes pulmonary dysfunction because of mutations in the transmembrane conductance regulator (CFTR) gene that disturb the cAMP-regulated Cl^- channel in airway epithelia (Zabner, J. et al., *Nature Genetics* 6:75-83, 1994). Adenovirus vectors engineered to carry the CFTR gene have been developed (Rich, D. et al., *Human Gene Therapy* 4:461-476, 1993) and studies have shown the ability of these vectors to deliver CFTR to nasal epithelia of CF patients (Zabner, J. et al., *Cell* 75:207-216, 1993), the airway epithelia of cotton rats and primates (Zabner, J. et al., *Nature Genetics* 6:75-83, 1994), and the respiratory epithelium of CF patients (Crystal, R.G. et al., *Nature Genetics* 8:42-51, 1994).

One of the critical issues remaining in the development of safe viral vectors is to prevent the generation of replication-competent virus during vector production in a packaging cell line or during gene therapy treatment of an individual. The generation of these replication competent viruses poses the threat of an unintended virus infection with attendant pathological consequences for the patient.

The presence of wild-type adenovirus in the recipient cells of human candidates for gene therapy presents a possibility for generating replication-competent adenovirus (RCA) due to homologous DNA

sequences present in the vector and the recipient cells (Jolly, D., *Cancer Gene Therapy* 1:51-64, 1994). Furthermore, the generation of new viruses carrying a transgene may interfere with dosage requirements for optimal gene therapy as extra copies of the gene may be produced by new viruses carrying the transgene. It is therefore critical to develop vectors that are not only replication-defective, but are designed to minimize recombinogenic potential as well limit the harmful effects of a recombination event by self-destruction.

Summary Of The Invention

This invention provides for gene therapy vectors that are effective to deliver useful genes to patients and which are constructed to minimize toxic or immunologic consequences to the patient.

The invention is directed to novel adenovirus vectors which are inactivated by the occurrence of a recombination event within a packaging cell or a recipient cell and therefore prevent the generation of replication-competent adenovirus (RCA). The inactivation may occur through the loss of an essential gene, or by the generation of a vector genome that cannot be packaged.

The invention is also directed to vectors which minimize the occurrence of a recombination event with packaging cells or recipient cells by vector genome rearrangements that decrease homology with viral sequences that may be present in a packaging cell or a recipient cell in order to prevent the generation of RCA.

These vector designs increase the safety of recombinant adenovirus vectors for use as gene transfer vehicles in gene therapy applications.

Thus, in one aspect, the invention provides a nucleotide sequence which contains elements of an

adenovirus genome as well as a heterologous gene of mammalian origin that is under the control of a eucaryotic transcriptional promoter. This nucleotide sequence is capable of functioning as a vector which allows expression of the aforementioned heterologous gene when the vector is placed in a cell of an individual. The said nucleotide sequence is further characterized by the absence from the sequence of a first element of the adenovirus genome that is essential to replication or packaging of the adenovirus in a host mammalian cell and the placement of a second element of the adenovirus genome that is itself essential to the replication or packaging of adenovirus in a host mammalian cell into the nucleotide sequence at, or directly adjacent to, the location the nucleotide sequence otherwise occupied by the first element.

An additional aspect of the invention is a nucleotide sequence where the first element is the E1a-E1b region of adenovirus genome and the second element may be any one of the E4 region of adenovirus, the region E2A, the gene encoding terminal protein or adenovirus structural proteins, such as fiber L5.

A still further aspect provides a nucleotide sequence containing elements of an adenovirus genome and a heterologous gene of mammalian origin that is under the control of a eucaryotic transcriptional promoter, in which the E1a-E1b region of the adenovirus genome is absent and where a stuffer sequence has been inserted into the nucleotide sequence in a location other than that of the heterologous gene of mammalian origin. A vector containing this sequence is further characterized in that legitimate recombination of the sequence with an element that is present in a helper cell used to replicate or package the sequence, or with an element that is present in a cell of an individual,

and having homology with the E1a-E1b region, leads to the production of a lengthened nucleotide sequence that is substantially less efficient than an unmodified nucleotide sequence at being packaged in the helper cell or in a cell of said individual.

The invention also provides for a nucleotide sequence, as above, that includes the gene for adenoviral protein IX and a heterologous gene of mammalian origin that is under the control of a eucaryotic transcriptional promoter. This latter nucleotide sequence is characterized in that the E1a-E1b region of the adenovirus genome is absent and the gene that encodes protein IX has been repositioned to a location that deviates from its normal location in the wild-type adenovirus genome.

The invention further provides for a nucleotide sequence, as above, that deletes the gene for adenoviral protein IX and includes a heterologous gene of mammalian origin that is under the control of a eucaryotic transcriptional promoter. This nucleotide sequence is also characterized in that the E1a-E1b region of the adenovirus genome is absent, and that the sequence does not exceed about 90% of the length of the adenovirus genome.

The invention also provides for a method for minimizing exposure of an individual undergoing gene therapy, using a virus vector to deliver a heterologous gene, to replication-competent virus comprising the step of treating said individual with a gene therapy composition that itself comprises a pharmaceutically acceptable carrier, and one or another of the vectors having the nucleotide sequences described above.

Brief Description Of The Figures

Fig. 1 Schematic diagram of current vector constructs, and the depiction of a recombination event in 293 cells. New constructs are depicted that produce a replication-incompetent vector by the deletion of an essential gene following recombination.

Fig. 2 A novel vector of the invention is depicted which, upon recombination with wild-type virus, produces replication-incompetent vectors deleted for an essential gene or segment.

Fig. 3 The 3' end of a novel vector is depicted, in which protein IX is repositioned to the E4-deleted region so as to minimize recombination between a vector and 293 cells.

Figs. 4A-D Comparison of the DNA sequences of adenovirus serotypes 2 and 5 from nucleotide 1-600 (Adenovirus type 2: SEQ ID NO: 1 and Adenovirus type 5: SEQ ID NO: 3) and 3041-4847 (Adenovirus type 2: SEQ ID NO: 2 and Adenovirus type 5: SEQ ID NO:4). The adenovirus 2 sequence is shown on the top line and the adenovirus 5 sequence is shown on the bottom line.

Fig. 5 Schematic diagram of various adenovirus vectors deleted for the E1 region and containing the CFTR gene cloned into the E1 site in the adenovirus genome. The CFTR gene is under the control of a specific eucaryotic transcriptional promoter and polyA site as illustrated in each vector. Additional alterations of

the adenovirus genome in each vector are shown.

Fig. 6 BclI restriction enzyme analysis of wild-type adenovirus serotypes 2 and 5 and of the adenovirus vectors shown in Figure 5. The restriction enzyme pattern of RCA generated during vector production in 293 cells is shown below each vector.

Fig. 7 Schematic diagram of RCA generated during vector production in 293 cells. The structure of RCA is shown with reference to the specific nucleotide borders of the recombination site and to the serotype source of the E1 region and the protein IX gene.

Figs. 8A-B Schematic diagram of the construction of pAd2/E1ACFTRsvdra-.

Fig. 9 Schematic diagram of the construction of pAdE4ORF6ΔE3B.

Fig. 10 Schematic diagram of in vivo recombination steps used to produce Ad2/CFTR-7.

Fig. 11 Schematic diagram of experiments to assay RCA generation during multiple passages of adenovirus vectors in 293 cells. The schedule of passages is shown along with the RCA bioassay performed after passages 3, 6, 9 and 12. HA refers to the HeLa and A549 cells used sequentially in the assay; the 2 numbers following indicate the number of days, respectively, of infection in each cell line. The infective dose used in the RCA assay is shown where E=exponent, and is expressed in infectious units (IU).

Detailed Description Of The Invention

The invention is directed to adenovirus vectors which are inactivated by the occurrence of a legitimate recombination event within a packaging cell or a recipient cell and therefore prevent the generation of replication-competent adenovirus (RCA). Legitimate recombination is that which is dependent on specific and normal base pairing at sequences recognized as having homology for each other. The inactivation may occur through the loss of an essential gene, or by the generation of a vector genome that cannot be packaged.

The invention is also directed to vectors which minimize the occurrence of a recombination event with packaging cells or recipient cells by vector genome rearrangements that decrease homology with viral sequences that may be present in a packaging cell or a recipient cell to prevent the generation of RCA. Recipient cells targeted for gene therapy may contain wild-type adenovirus DNA sequence that can recombine with an adenovirus vector (Jolly, D., *Cancer Gene Therapy* 1:51-64, 1994).

These vector designs therefore increase the safety of recombinant adenovirus vectors for use as gene transfer vehicles in gene therapy applications.

Thus, in one aspect, the invention provides a nucleotide sequence which contains elements of an adenovirus genome as well as a heterologous gene of mammalian origin that is under the control of a eucaryotic transcriptional promoter. This nucleotide sequence is capable of functioning as a vector which allows expression of the aforementioned heterologous gene when the vector is placed in a cell of an individual. The nucleotide sequence is further characterized by the absence from the sequence of a first element of the adenovirus genome that is essential to replication or packaging of the adenovirus in a host mammalian cell and the placement of a second

element of the adenovirus genome that is itself essential to the replication or packaging of adenovirus in a host mammalian cell into the nucleotide sequence at, or directly adjacent to, the location the nucleotide sequence otherwise occupied by the first element.

It is understood according to the practice of the invention that the reference to elements of the viral genome (such as first and second elements, referred to herein) that are termed essential includes also reference to elements that facilitate replication or packaging but which are not absolutely essential to such processes.

With respect to this aspect of the invention, the heterologous gene is any gene which is recognized as useful. Representative examples include genes of mammalian origin encoding, for example, proteins or useful RNAs; viral proteins such as herpes thymidine kinase, and bacterial cholera toxin for cytotoxic therapy.

An additional aspect of the invention is a nucleotide sequence where the first element is the E1a-E1b region of adenovirus genome and the second element may be any one of the E4 region of adenovirus, the region E2A, the gene encoding terminal protein or adenovirus structural proteins, such as fiber L5.

A still further aspect provides a nucleotide sequence containing elements of an adenovirus genome and a heterologous gene of mammalian origin that is under the control of a eucaryotic transcriptional promoter, in which the E1a-E1b region of the adenovirus genome is absent and where a stuffer sequence has been inserted into the nucleotide sequence in a location other than that of the heterologous gene of mammalian origin. A vector containing this sequence is further characterized in that legitimate recombination of the

sequence with an element that is present in a helper cell used to replicate or package the sequence, or with an element that is present in a cell of an individual, and having homology with the E1a-E1b region, leads to the production of a lengthened nucleotide sequence that is substantially less efficient than an unmodified nucleotide sequence at being packaged in the helper cell or in a cell of said individual.

By additional sequence it is meant an inert sequence which does not affect adversely the function of the vector. The length of the additional sequence is selected based on the length of the sequence deleted. For example, if the deletion consists of the E1 region, an acceptable insert is about 3 kb, which is based on principles known by those skilled in the art, based on consideration of vector length for optimal packaging.

The invention also provides for a nucleotide sequence, as above, that includes the gene for adenoviral protein IX and a heterologous gene of mammalian origin that is under the control of a eucaryotic transcriptional promoter. This latter nucleotide sequence is characterized in that the E1a-E1b region of the adenovirus genome is absent and the gene that encodes protein IX has been repositioned to a location that deviates from its normal location in the wild-type adenovirus genome.

Preferably, it is repositioned to a location of generally at least about 100 nucleotides removed, preferably about 500 nucleotides removed, and most preferably, about 100 nucleotides removed.

The invention also provides for a nucleotide sequence, as above, that deletes the gene for adenoviral protein IX and includes a heterologous gene of mammalian origin that is under the control of a eucaryotic transcriptional promoter. This nucleotide

sequence is also characterized in that the E1a-E1b region of the adenovirus genome is absent, and that the sequence does not exceed about 90% of the length of the adenovirus genome.

The invention also provides for a method for minimizing exposure of an individual undergoing gene therapy, using a virus vector to deliver a heterologous gene, to replication-competent virus comprising the step of treating said individual with a gene therapy composition that itself comprises a pharmaceutically acceptable carrier, and vectors using the nucleotide sequences described above.

Recombination-Dependent
Target Sequence Deletion Vectors

This aspect of the invention relates to vectors that prevent the generation of RCA by an adenovirus vector design in which an essential gene or genomic segment (the deletion target) is placed within a region that is potentially subject to recombination because a packaging cell or recipient cell contains homologous viral sequences. The result of a potential recombination event between cellular sequences and the vector is that this essential gene or genomic segment is deleted upon recombination, thereby rendering the viral vector replication-incompetent. This is accomplished by rearranging the genome so that the deletion target is moved from its original genomic location to be located within the region potentially subject to recombination. Although recombination may restore a missing viral sequence, the virus will be impaired by the loss of an essential gene that is caused by the recombination event.

In one embodiment of the invention, this vector design is applicable to preventing recombination events in a packaging cell line, such as 293 cells (Graham,

F.L., *J. Gen. Virol.* 36:59-72, 1977). These cells, which contain an intact contiguous viral E1 DNA sequence derived from adenovirus 5 from the 5' ITR to about nucleotide 4300 (ref. for numbering is Roberts, R.J., in *Adenovirus DNA*, Oberfler, W., ed., Matinus Nihoft Publishing, Boston, 1986) integrated into the genome, are able to supply the E1 gene products in trans to an E1-deleted adenovirus vector. The generation of RCA is possible from recombination between the E1 sequences in the cell and the remaining sequences at the boundary of E1 in the vector, such as protein IX, if enough flanking homologous sequence is present to facilitate a legitimate recombination event.

In a specific embodiment, an adenovirus vector deleted for the E1 region and the E4 region except for the ORF6 gene is constructed by inserting an expression cassette into the E4-deleted region. (Fig. 1). The ORF6 gene is moved to the E1-deleted region. The E4 region of an adenovirus vector may be deleted except for ORF6 due to its role in DNA replication, late mRNA accumulation, and shutoff of host protein synthesis (Bridge, E. et al., *J. Virol.* 63:631-638, 1989; Huang, M. et al., *J. Virol.* 63:2605-2615, 1989). If a recombination event occurs between the viral sequences and 293 cells, the E1 sequences are gained and the ORF6 gene is deleted, such that the vector is still replication-defective.

In a further aspect of the invention, a vector may be customized to prevent the generation of RCA from any packaging cell line. The deletion target gene or segment will be engineered into the region of the vector which has homology with the DNA contained in the packaging cell line. Thus, recombination within this region will cause the target gene or segment to be deleted, resulting in the generation of replication-incompetent viral vectors. Vectors in

which the deletion target is inserted into the E2 or E4 regions, for example, may be designed to circumvent recombination events in packaging cell lines that supply E2 or E4 gene products (Klessig, D. et al., *Mol. Cell. Biol.* 4:1354-1362, 1984; Weinberg, D. et al., *PNAS* 80:5383-5386, 1983). Analogous constructs designed to circumvent recombination in analogous packaging cell lines are within the scope of the invention.

In a further embodiment of this invention, this vector design can be used to preclude the formation of RCA from recombination with wild-type adenovirus that may be present in a patient's cell. The presence of wild-type adenovirus in human candidates for adenovirus-based gene therapy may present a source of viral DNA sequences for recombination events that generate RCA from a replication-incompetent adenovirus vector (Jolly, D., *Cancer Gene Therapy* 1:51-64, 1994). Prevention of RCA production may be accomplished by placing essential genes or segments within one or more regions in the vector that may potentially be subject to recombination with the wild-type adenovirus. By placing essential targets in potential sites for recombination, one or more recombination events will serve to delete essential viral genes, and thereby render the viral vector replication-incompetent.

In another embodiment, depicted in Fig. 2, a vector is constructed that upon recombination with wild-type virus, is rendered replication-incompetent. The vector contains the ORF6 gene positioned in the deleted E1 region, and an expression cassette inserted into the deleted E4 region. The central portion of the vector genome is homologous to wild-type adenovirus, and upon a recombination event, the vectors genomes so generated will be replication-incompetent as depicted in Fig. 2.

Essential adenovirus genes or genomic segments which may be positioned to serve as targets for deletion upon a recombination event include ORF6, L5 (fiber protein), the entire E4 region, the E2A region, terminal protein, or any other essential viral genes or segments.

Recombination-Dependent
Packaging-Defective Vectors

This aspect of the invention relates to vectors that are rendered packaging-defective upon the occurrence of a recombination event with a packaging cell or a recipient cell, preventing the generation of RCA. This design takes advantage of limitations that exist on the genome length that can be packaged into an adenovirus virion. The size of an adenovirus genome that can be optimally packaged into new virions may exceed its wild-type length up to about 105%-108% and still be packaged into new virions (Berkner, K.L., *Curr. Top. Micro. Immunol.* 158:39-66, 1992). If a recombination event generates a virus genome that exceeds the packaging limit, it will not be packaged and RCA are not generated.

Vectors that are packaging-defective following recombination can be created by engineering the vector DNA such that its length is at least 101% of the wild-type length. This can be accomplished even with vectors that contain deletions of the wild-type adenoviral genome because of the insertion of a heterologous DNA sequence that compensates for the deletion and maintains the genome at near-wild-type length.

The heterologous DNA sequence may solely code for a gene of interest, or alternatively, where a heterologous gene is at small size, additional heterologous stuffer DNA sequence may be added so as to

render the vector genome at a size of at least 101% of wild-type length. Stuffer is a term generally recognized in the art intended to define functionally inert sequence intended to extend the length, thereof, such as certain portions of bacteriophage lambda.

In another embodiment of this aspect of the invention, a vector is designed in which the E1 region is deleted as well as the E4 region except for the ORF6 gene, for a total deletion of 5 kb, and the CFTR gene is inserted into the E4 deletion region. This vector size is 101.3% of wild-type length. Following an E1-mediated recombination event in 293 cells, for example, that inserts the E1 region into the vector, the genome will increase to about 108% of wild-type length, rendering it packaging-defective and preventing the generation of RCA.

It will be understood by those skilled in the art that the concept of recombination-dependent packaging-defective adenovirus vectors may be practiced by using any number of viral or non-viral DNA fragments that are engineered into any number of sites in the vector, with an overall goal of maintaining a vector size that will exceed optimal packaging length upon recombination.

Scrambled Genome Vectors That Minimize Recombination And Generation Of RCA By Recombination

In this aspect of the invention, the vector genome derived from wild-type adenovirus is rearranged so as to perturb the linear arrangement of the viral coding regions. In one embodiment, this "scrambling" of the genome reduces the potential for recombination between a wild-type adenovirus that may be found in a human candidate for gene therapy and the adenovirus vector. This reduction is due to the fact that long stretches

of homologous DNA sequences between the cell and vector are eliminated when the viral sequences in the vector are rearranged. The likelihood of recombination is reduced as the homologous regions are reduced in length. In this manner, the generation of RCA is minimized. Regions of the adenovirus genome which may be scrambled included, for example, the E2A region, the E4 region, ORF6, L5 (fiber protein), terminal protein, or any combination of these and other regions of the viral genome which result in a scrambled genome whose linear sequence deviates from wild-type.

This concept may be applied to vectors where more than one region of the adenovirus is deleted, such that restoration of replication-competence requires several recombination events, each of which is rendered less likely as the linear homology between the vector and cell is reduced by scrambling.

This concept may be analogously applied to minimizing recombination between an adenovirus vector and a packaging cell line, by designing the vector so that stretches of homology with the cell line are perturbed by rearrangement, reducing their effective length and the likelihood of recombination. In one example of this embodiment of the invention, the potential for recombination between an adenovirus vector and 293 cells is decreased by rearranging the protein IX sequences in the vector. The protein IX sequences are often found at the right-hand boundary of the deleted E1 region in a vector. Protein IX sequences are also contained within 293 cells at the boundary of the E1 adenovirus insert, and may facilitate recombination between the vector and cellular sequences. The result is that restoration of E1 sequences to the vector may occur by a protein IX-mediated recombination event. The relocation or mutagenesis of a protein IX boundary from the E1

deletion region in a vector will decrease the likelihood of such an event, and of the generation of RCA. Such a vector is described in Example 1, *infra*, and Fig. 3.

Ad2/CFTR-8 is particular embodiment of this aspect of the invention, and is shown in Figure 5.

Prevention Of RCA With Vectors
Deleted For Homology With Packaging Cell Lines

This aspect of the invention relates to vector designs that prevent the generation of RCA during vector production by deletion of recombinogenic DNA sequences. RCA generation may occur during vector production when regions of homology exist between the viral DNA sequences in a replication-incompetent deletion vector and the viral DNA sequences in a packaging cell line that supplies viral proteins in trans. The vectors in this embodiment of the invention are designed such that regions of homology between the viral genome and the packaging cell line are further minimized by the deletion of non-essential viral DNA. These vectors are pared down to minimal viral sequences required to accomplish the goal of transporting a gene of interest into the target cell and presenting the gene to the cell for expression, but designed so that maximal safety is accomplished by preventing RCA formation.

Adenovirus DNA sequences that have been deleted in vector designs to date include sequences from the E1, E3 and E4 regions of the viral genome (Berkner, K.L., Curr. Top. Micro. Immunol. 158:39-66, 1992). The present invention provides vectors in which the protein IX region of the viral genome has been deleted so as to further reduce any homology with a packaging cell line containing adenovirus sequences. This deletion is particularly useful when vectors are being packaged in

a cell line that includes protein IX sequences in the viral insert in the cell genome. For example, the 293 cell line widely used in adenovirus vector production contains the E1 regions and the protein IX sequence derived from adenovirus serotype 5 (Graham, F.L., J. Gen. Virol. 36:59-72, 1977), and is permissive for the growth of E1-deletion vectors.

A particular vector of the present invention, Ad2/CFTR-7, was constructed so as to delete the viral gene encoding protein IX. This gene is found at the right hand boundary of the E1B region and encodes a protein which is involved in packaging of full-length genomes during virion assembly (Ghosh-Choudhury, G. et al., J. EMBO 6:1733-1739, 1987). The protein IX DNA sequence in a vector has the potential for recombination with protein IX sequences contained within the adenovirus E1 insert in the 293 cell line. Because such a recombination event may generate RCA during the course of vector production, the vector described here provides a means to avoid this possibility by the removal of the protein IX recombinogenic sequences.

The removal of the protein IX gene is tolerated by a vector design that reduces the amount of DNA to be packaged, since protein IX is required to package genomes which are at least 90% of wild-type length (Ghosh-Choudhury, G. et al., J. EMBO 6:1733-1938, 1987). This may be accomplished by deletions of nonessential sequences, or by the deletion of sequences which are not necessary in cis, and whose gene products may be supplied in trans. Such sequences include those derived from the adenovirus E1, E3 and E4 regions of the genome. In Ad2/CFTR-7, the E3 region was reduced in order to reduce genome length. It may be desirable to reduce the viral genome size with E3 deletions, yet retain some E3 sequences due to the fact that E3

proteins are involved in minimizing host immune response to adenovirus proteins (Horwitz, M.S., Adenoviridae and their Replication, in Virology, 2nd. ed., Fields, B.N. et al., eds., Raven Press, New York, 1990). In this manner, untoward consequences of viral vector introduction into a patient may be prevented.

The ability of an adenovirus vector design to minimize the potential for RCA generation can be assessed by determining the RCA level in a cycle of vector production using a bioassay. The assay scores for RCA generated during vector production by using cell lines that are not permissive for replication-incompetent deletion vectors and will only support the growth of wild-type adenovirus. These cell lines are infected with a vector stock, and the presence or absence of an observable cytopathic effect (CPE) is used to score for any generation of RCA.

Where an adenovirus deletion vector which is replication-incompetent has been packaged in a cell line that contains adenovirus sequences supplying essential viral proteins in trans, RCA generated from a recombination event contains a mixture of viral DNA sequences from both sources. Such a hybrid genome in the RCA may be characterized when the viral sequences in the cell line and the vector are derived from different virus serotypes. In this manner, the sequence heterogeneity among virus serotypes may be used to identify a recombination event by any number of techniques known to those skilled in the art, such as restriction enzyme analysis or direct DNA sequencing. Comparison of sequenced regions in the RCA to the known sequence of the adenovirus serotypes allows for identification of the source of the sequences tested. Thus, the recombination event giving rise to the RCA can be dissected by sequence analysis.

A specific example of using RCA genome analysis to identify the nature of the recombination event can be shown using adenovirus vectors deleted for the E1 region and in which the gene of interest is cloned into the E1 site. These vectors are produced in 293 cells. Where the vector is produced from an adenovirus serotype that is different than that used to construct the 293 cell line, e.g., adenovirus 2, any RCA that is generated by recombination between the adenovirus 5 sequences in the cell and the adenovirus 2 sequences in the vector can be characterized by different restriction enzyme patterns between the 2 serotypes. Furthermore, DNA sequencing can be used to identify specific sequence variations. When E1-deletion vectors are used, any RCA generated from a recombination event will incorporate the E1 region from the adenovirus 5 insert in the 293 cells, and the presence of these sequences in the RCA can be identified by characterization of the E1 region. The E1 region of the RCA can be mapped by restriction enzyme analysis and/or sequenced directly to determine the origin of this sequence. Therefore, the skilled artisan can confirm that the RCA contains a mixture of adenovirus 2 and adenovirus 5 sequences, indicating that a recombination event occurred between the cell and vector viral DNA sequences.

While vectors deleted for protein IX have particular relevance to the prevention of RCA during vector production in packaging cell lines that contain protein IX sequences - i.e., 293 cells - it may be understood by those skilled in the art that the concept of using gene or sequence deletion may be analogously extended to the design of vectors that minimize or delete any regions of viral sequences when used in cell lines that contain homologous viral sequences and therefore have the potential to generate RCA.

Parameters Of The Vectors

The adenovirus vectors of the invention may be derived from the genome of various adenovirus serotypes, including but not limited to, adenovirus 2, 4, 5, and 7, and in general, non-oncogenic serotypes.

The adenovirus vectors of the invention may be engineered to carry any heterologous gene for delivery and expression to a target cell. The gene may be engineered into various sites within the vectors, including but not limited to, the E1 region, the E2 region, the E3 region and the E4 region, using techniques that are well known to those skilled in the art (*Current Protocols in Molecular Biology*, Ausubel, F. et al., eds., Wiley and Sons, New York, 1995). The heterologous gene cloned into the adenovirus vector may be engineered as a complete transcriptional unit, including a suitable promoter and polyadenylation signal. Such promoters including the adenovirus E1 promoter or E4 promoter, for example, as well as others including, but not limited to, the CMV promoter and the PGK promoter. Suitable polyadenylation signals at the 3' end of the heterologous gene include, but are not limited to, the BGH and SV40 polyadenylation signals. The E3 region of the adenovirus genome may be deleted in order to increase the cloning capacity of a vector, or it may be left in the vector construct, according to conditions encountered by one practicing the present invention. It is presently preferred to leave at least a substantial portion of the E3 region in the vector so as to minimize, in some aspects, immune response by the patient to the vector construct, including serious inflammatory consequences.

Genes that may be engineered into the adenovirus vectors of the invention include, but are not limited to, CFTR for CF, α1-antitrypsin for emphysema, soluble CD4 for AIDS, ADA for adenosine deaminase deficiency

and any other genes that are recognized in the art as being useful for gene therapy.

The vectors of the present invention may have application in gene therapy for the treatment of diseases which require that a gene be transferred to recipient cells for the purpose of correcting a missing or defective gene, or for the purpose of providing a therapeutic molecule for treatment of a clinical condition.

The vectors of the present invention can be adapted to *ex vivo* and *in vitro* gene therapy applications.

It will be understood that the concepts of vector designs contained in the foregoing sections may analogously be applied to other viral vectors, including, but not limited to, retrovirus, herpes, adeno-associated virus, papovavirus, vaccinia, and other DNA and RNA viruses.

Example 1: CONSTRUCTION OF A SCRAMBLED ADENOVIRUS VECTOR THAT PREVENTS PROTEIN IX-DEPENDENT RECOMBINATION

A novel adenovirus vector is constructed by starting with the plasmid Ad2E4ORF6 (PCT Publication Number WO 94/12649), deleted for E1 and in which E4 sequences are deleted from the ClaI site at 34077 to the TaqI site at 35597. The ORF6 sequence from 33178 to 34082 is inserted into the E4 region. The SV40 early polyA sequence is inserted adjacent to the ORF6, which also serves to prevent readthrough from the ORF6 gene into the L5 (fiber) sequences. Protein IX is repositioned from its original location in the virus genome into the E4-deleted region as a Bam HI fragment. The protein IX fragment contains its own promoter, and may be cloned into the vector in either direction. The construct is shown in Fig. 3. The plasmid is

transfected into 293 packaging cells to produce a vector stock using standard techniques (*Current Protocols in Molecular Biology*, Ausubel, F., et al., eds., Wiley & Sons, 1995). The resulting vector is less susceptible to a recombination event with viral sequences in 293 cells due to the repositioning of the protein IX gene, which decreases homology between the vector and the 293 cell.

Ad2/CFTR-8 is an example of an adenovirus vector in which protein IX has been repositioned into the E4 region of the virus genome, and is shown in Figure 5.

EXAMPLE 2: ANALYSIS OF RCA BY SEROTYPE SEQUENCE HETEROGENEITY

The generation of RCA arising from recombination between an adenovirus vector and 293 cells was analyzed by sequence analysis of replication-competent virus that arose during vector production. The vectors were derived from adenovirus serotype 2 and were deleted for the E1 region, but contained the protein IX sequence. The 293 cells contain the E1 region and the protein IX sequence from adenovirus serotype 5. Sequence heterogeneity between adenovirus serotypes 2 and 5 was used to identify the source of E1 and protein IX sequences that were contained in the RCA. If the protein IX sequence in the RCA is derived from adenovirus 5, then a homologous recombination event between the vector and the 293 cells can be scored. Sequence heterogeneity between these adenovirus serotypes from nucleotide 1-600 (Adenovirus type 2: SEQ ID NO: 1 and Adenovirus type 5: SEQ ID NO: 3) and 3041-4847 (Adenovirus type 2: SEQ ID NO: 2 and Adenovirus type 5: SEQ ID NO: 4) is shown in Figures 4A-D.

The vectors analyzed for RCA generation during production are shown in Figure 5. Figure 6 shows the results of BclI restriction enzyme analysis of each

vector and of the RCA generated during vector production in 293 cells. By reference to the restriction sites in the wild-type adenovirus 2 and 5 serotypes, the RCA can be characterized with respect to the source of its sequences. In such a manner, the recombination event between a vector and a packaging cell line that gives rise to RCA may be identified. Figure 7 provides a schematic diagram of the sequence analysis of the RCA generated during production of each vector in 293 cells. The adenovirus 5 sequences contained in 293 cells, which appear at the top of each schematic, are potentially available for a recombination event with the protein IX sequence in the vector. The figure shows the recombination sites at the 5′ and 3′ ends of the E1 insert in the RCA for each vector tested. In RCA generated during production of vectors Ad2/CFTR-2, Ad2/CFTR-5 and Ad2/CFTR-6, the protein IX sequence at the 3′ boundary of the E1 fragment in the RCA is derived from adenovirus 5, indicating that a recombination event occurred between the vector and the 293 cells, mediated by the protein IX sequence. The results from Ad2/CFTR-3 and Ad2/CFTR-1 were variable, and recombination that was not mediated by protein IX was detected.

The results of the recombination analysis of the RCA demonstrates that the protein IX sequence in an adenovirus vector can serve as a recombinogenic site for the generation of RCA in a cell line that contains a homologous protein IX sequence.

EXAMPLE 3: CONSTRUCTION AND ANALYSIS OF Ad2/CFTR-7

A series of cloning steps was required to construct the plasmids intermediate to the final construction of vector Ad2/CFTR-7. The *in vivo* recombination steps to derive Ad2/CFTR-7 are detailed below. An RCA assay was used to determine whether the

Ad2/CFTR-7 vector design reduced RCA generation during passage in 293 cells.

Construction of pAd2/E1aCFTRsvdra-

The cloning steps and plasmids used in constructing the intermediate plasmid pAd2/E1aCFTRsvdra- are described below are illustrated in Figures 8A and 8B. The starting plasmid, pAd2/CMV-2, contains an insert of approximately 7.5 kb cloned into the ClaI and BamHI sites of pBR322 which comprises the first 10,680 nucleotides of Ad2, except for a deletion of sequences between nucleotides 357 and 3498. This deletion eliminates the E1 promoter, E1a and most of E1b coding region. Plasmid pAd2/CMV-2 also contains a CMV promoter inserted into the ClaI and SpeI sites at the site of the E1 deletion and a downstream SV40 polyadenylation (polyA) sequence (originally a 197 bp BamHI-BclI fragment) cloned into the BamHI site.

The first series of cloning steps first deleted a portion of the SV40 polyA and a portion of the protein IX gene and subsequently the remainder of the protein IX gene. Plasmid pAd2/CMV-2 was digested with SpeI and HindIII. The 3146 bp fragment containing the SV40 polyA and Ad2 sequences was ligated into the same sites of pBluescript SK- (Stratagene) to produce plasmid pBSSK/s/h. The 656 bp MunI fragment containing 60 nucleotides of the SV40 polyA and the majority of the protein IX sequences of Ad2 was excised from this plasmid to produce plasmid PBS-SH mun-. This plasmid was digested with DraI and HindIII and the 2210 bp fragment was cloned into the EcoRV and HindIII sites of pBluescript SKII- (Stratagene) resulting in plasmid pBSDra-HindIII. In this step, the remainder of the protein IX gene was removed. The EcoRI - HindIII fragment (2214 bp) of this plasmid was then cloned into the MunI and HindIII sites of plasmid pBS-SHmun- producing pBS-SH.dra-. In this step, the segment of

the Ad2 genome with the protein IX deletion is rejoined with the truncated SV40 polyA segment. Plasmid pBS-SH.dra- thus has a 60 bp deletion of SV40 polyA, a deletion of the protein IX gene, and Ad2 sequences from bp 4020 through 10680. This insert is also surrounded by polylinker sites.

In the next series of cloning steps, the DNA segment produced above containing the SV40 polyA and the protein IX deletion was joined with sequences required to complete the left end of the Ad2 genome. pBS-SH.dra- was digested with AvrII and HindIII and the 2368 bp fragment was cloned into the AvrII and HindIII sites of plasmid pAdE1aBGH, effectively replacing the BGH polyA, protein IX and Ad2 sequences from this plasmid and thus producing plasmid pAd2/E1asvdra-.

In the next series of cloning steps, the CFTR CDNA was introduced downstream from the E1A promoter in pAd2/E1asvdra-. To accomplish this a SwaI and AvrII fragment containing the CFTR CDNA was released from plasmid pAdPGKCFTRsv and inserted into the SwaI and AvrII sites of pAd2/E1asvdra- to produce plasmid pAd2/E1aCFTRsvdra-. This plasmid was used in the *in vivo* recombination described below.

Construction of pAd2/ORF6E3Δ1.6

The cloning steps and plasmids for preparing pAD2/ORF6E3Δ1.6 are detailed in Figure 9. The starting plasmid, pAdE40RF6, was described in PCT Publication Number WO 94/12649. The 1.6 kb deletion within the E3 region of this plasmid was constructed by three-way ligation of two PCR fragments into MluI and EagI cut pAdE40RF6. The PCR fragments were both made using pAdE40RF6 DNA and the first PCR fragment corresponded to Ad2 nucleotides 27123 through 29292 (2169 bp) and was flanked by EagI and RsrII sites respectively. The second PCR fragment corresponded to Ad2 nucleotides 30841 through 31176 (339 bp) and was flanked by RsrII

and MluI sites respectively. When ligated with MluI and EagI cut pAdE40RF6 DNA the resulting plasmid pAdORF6Δ1.6 contained a deletion of Ad2 nucleotides 29293 through 30840 (1547 bp) or all of E3b except for the polyA site. It retained the rest of the Ad2 sequences from 27123 through 35937 and also now contains a unique RsrII site.

In vivo Recombination Steps Used to Derive Ad2/CFTR-7

The recombination steps used to derive the DNA construct of Ad2/CFTR-7 are illustrated in Figure 10.

Plasmid pAd2E40RF6Δ1.6 linearized with ClaI (polylinker region of plasmid past Ad2 bp 35937) and Ad2 DNA digested with PacI (bp 28622 of Ad2) and AseI (multiple cuts 3' of PacI) were introduced into 293 cells using $CaPO_4$ transfection. The desired recombinant virus resulting from this step, AdORF6Δ1.6, was plaque purified and used to produce a seed stock. Next, pAd2/ElaCFTRsvdra- was cleaved with BstBI at the site corresponding to the unique BstBI site at 10670 in Ad2. Genomic DNA from Ad2/ORF6E3Δ1.6 was digested with PshAI which cleaves twice in the 5' region of the virus. Plasmid and genomic DNAs were then transfected with $CaPO_4$ (Promega) into 293 cells. The desired recombinant vector resulting from this step, Ad2/CFTR-7, was plaque purified and used to produce a seed stock. Ad2/CFTR-7 is shown in Figure 5.

EXAMPLE 4: RCA ASSAY OF VECTORS PASSAGED IN 293 CELLS

The Ad2/CFTR-7 vector was tested to determine if RCA generation arose during blind passages when compared with other vectors in which the protein IX region is retained. An RCA bioassay was used to score for RCA. A schematic diagram of the RCA assay design is shown in Figure 11.

A schematic diagram of the vectors tested is shown in Figure 5. The vectors tested in comparison to Ad2/CFTR-7 include Ad2/CFTR-1, Ad2/CFTR-2, and Ad2/CFTR-6. All of these control vectors contain the protein IX gene.

A seed stock of each vector was prepared by growth of the virus in 293 cells, which contain the adenovirus E1 region and are permissive for the replication of E1-deletion vectors. The seed stock was titered on 293 cells.

Serial passaging of the seed stock was performed on 293 cells. An inoculum of virus at an M.O.I. (multiplicity of infection) of 5-10 was used to infect the cells. Each passage was harvested when the cytopathic effect (CPE) was observed to be 100%, and a lysate was prepared according to standard techniques.

The assay of RCA generation in 293 cells was tested by a bioassay for replication competent virus which was performed using HeLa cells and A549 cells. These cell lines do not contain any adenovirus E1 sequences, and are therefore only permissive for viruses which contain the E1 region by design or have acquired it by a recombination event. Therefore, the assay scores for any RCA generated from a recombination event between an E1-deleted vector and the 293 cells.

Selected passages of each vector through 293 cells were analyzed by the RCA assay. The assay was performed by infecting HeLa cells with the vector passage to be tested at an MOI of 20. This infection was allowed to proceed for 4 days, after which the cells were harvested and a lysate prepared by standard techniques. The lysate was then used to infect A549 cells, and this infection proceeded for 10 days. The cells were scored for the presence or absence of CPE. Table 1 sets forth the results of RCA assays performed on selected passages of each vector tested. A passage

was scored as PASS if no RCA was observed, and was scored as a FAIL if RCA was observed, as determined by any observation of CPE. The dose of vector tested in the RCA assay was varied, as shown.

The results from the RCA assay show that RCA was observable in passage 12 from vectors Ad2/CFTR-2 and Ad2/CFTR-6, and in passage 3 from vector Ad2/CFTR-1. In contrast, no RCA was observed at passage 12 from vector Ad2/CFTR-7, even at the highest dose tested. This vector has the lowest levels of RCA of the vectors tested. The results indicate that removal of the protein IX sequences has significantly reduced RCA generation in 293 cells.

TABLE 1

Adenovirus Vector	Seed Stock Titer (IU/ml)	Passage Titers (IU/ml)	Dose Tested in RCA Assay		
			1.25×10^8 IU	2.5×10^9 IU	2.0×10^{10} IU
Ad2/CFTR-1	1.0×10^8	P1: 2.2×10^9 P6: 3.6×10^9	P3, P6, P9, P12: PASS	P3: PASS P12: PASS	P3: FAIL P12: PASS
5 Ad2/CFTR-2	3.8×10^8	P1: 7.2×10^9 P6: 2.2×10^9	P3, P6, P9, P12: PASS	P3: PASS P12: FAIL w/4	P3: PASS P12: FAIL 100%
Ad2/CFTR-6	7.6×10^8	P1: 1.8×10^9 P7: 3.0×10^9	P3, P6, P9, P12: PASS	P3: PASS P12: PASS	P3: PASS P12: FAIL w/20
Ad2/CFTR-7	1.1×10^8	P1: 3.4×10^7 P7: 1.9×10^8	P3, P6, P9, P12: PASS	P3: PASS P12: PASS	P3: PASS P12: PASS

Results of the RCA assay performed on selected passages of each vector through 293 cells are shown. The seed stock titer and passage titers were performed on 293 cells. The RCA assay was performed as described in EXAMPLE 3. The observation of CPE in the assay was scored as a FAIL, while the absence of CPE was scored as a PASS.

EXAMPLE 5: ADENOVIRUS VECTORS WITH MINIMAL E4 SEQUENCE

Plasmid pAdE40RF6 was described in PCT Publication Number WO 04/12649 and used to construct Ad2-ORF6/PGK-CFTR, also described in the same publication. It contains the CFTR gene under the control of the PGK promoter. Ad2/CFTR-8, shown in Figure 5, is an adenovirus vector which is equivalent to Ad2-ORF6/PGK-CFTR.

Further modifications of this vector design are an aspect of the present invention. The CFTR gene may alternatively be placed under the control of the CMV promoter, as illustrated by Ad2/CFTR-5, as shown in Figure 5. Other promoters which can be used include the adenovirus major late promoter (MLP), as illustrated in the vector Ad2/CFTR-4. The BGH and SV40 polyA elements can be used in vector construction, as well as others known to those skilled in the art.

SEQUENCE LISTING

(1) GENERAL INFORMATION

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WADSWORTH, SAMUEL C.

(ii) TITLE OF THE INVENTION: NOVEL ADENOVIRUS VECTORS FOR GENE THERAPY

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 01701-9322

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/540,077
(B) FILING DATE: 06-OCT-1995
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: 08/409,874
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(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 35,492
(C) REFERENCE/DOCKET NUMBER: GEN5-1.1 PCT

(ix) TELECOMMUNICATION INFORMATION:
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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 600 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GTGTGCGCCG GTGTATACGG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG    240
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1796 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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TTCCTACCTT ACCAATGCAA TTTGAGTCAC ACTAAGATAT TGCTTGAGCC CGAGAGCATG    240
TCCAAGGTGA ACCTGAACGG GGTGTTTGAC ATGACCATGA AGATCTGGAA GGTGCTGAGG    300
TACGATGAGA CCCGCACCAG GTGCAGACCC TGCGAGTGTG GCGGTAAACA TATTAGGAAC    360
CAGCCTGTGA TGCTGGATGT GACCGAGGAG CTGAGGCCCG ATCACTTGGT GCTGGCCTGC    420
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 600 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GTGTGCGCCG GTGTACACAG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG    240
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AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA GGGCCGCGGG    360
GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TTCCGCGTTC    420
CGGGTCAAAG TTGGCGTTTT ATTATTATAG TCAGCTGACG TGTAGTGTAT TTATACCCGG    480
TGAGTTCCTC AAGAGGCCAC TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCCGAGCCGC    540
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1800 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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GTGTTTGAGC ATAACATACT GACCCGCTGT TCCTTGCATT TGGGTAACAG GAGGGGGGTG    180
TTCCTACCTT ACCAATGCAA TTTGAGTCAC ACTAAGATAT TGCTTGAGCC CGAGAGCATG    240
TCCAAGGTGA ACCTGAACGG GGTGTTTGAC ATGACCATGA AGATCTGGAA GGTGCTGAGG    300
TACGATGAGA CCCGCACCAG GTGCAGACCC TGCGAGTGTG GCGGTAAACA TATTAGGAAC    360
CAGCCTGTGA TGCTGGATGT GACCGAGGAG CTGAGGCCCG ATCACTTGGT GCTGGCCTGC    420
ACCCGCGCTG AGTTTGGCTC TAGCGATGAA GATACAGATT GAGGTACTGA AATGTGTGGG    480
CGTGGCTTAA GGGTGGGAAA GAATATATAA GGTGGGGGTC TTATGTAGTT TTGTATCTGT    540
TTTGCAGCAG CCGCCGCCGC CATGAGCACC AACTCGTTTG ATGGAAGCAT TGTGAGCTCA    600
TATTTGACAA CGCGCATGCC CCCATGGGCC GGGGTGCGTC AGAATGTGAT GGGCTCCAGC    660
ATTGATGGTC GCCCCGTCCT GCCCGCAAAC TCTACTACCT TGACCTACGA GACCGTGTCT    720
GGAACGCCGT TGGAGACTGC AGCCTCCGCC GCCGCTTCAG CCGCTGCAGC CACCGCCCGC    780
GGGATTGTGA CTGACTTTGC TTTCCTGAGC CCGCTTGCAA GCAGTGCAGC TTCCCGTTCA    840
TCCGCCCGCG ATGACAAGTT GACGGCTCTT TTGGCACAAT TGGATTCTTT GACCCGGGAA    900
CTTAATGTCG TTTCTCAGCA GCTGTTGGAT CTGCGCCAGC AGGTTTCTGC CCTGAAGGCT    960
TCCTCCCCTC CCAATGCGGT TTAAAACATA AATAAAAAAC CAGACTCTGT TTGGATTTGG   1020
ATCAAGCAAG TGTCTTGCTG TCTTTATTTA GGGGTTTTGC GCGCGCGGTA GGCCCGGGAC   1080
CAGCGGTCTC GGTCGTTGAG GGTCCTGTGT ATTTTTTCCA GGACGTGGTA AAGGTGACTC   1140
TGGATGTTCA GATACATGGG CATAAGCCCG TCTCTGGGGT GGAGGTAGCA CCACTGCAGA   1200
GCTTCATGCT GCGGGGTGGT GTTGTAGATG ATCCAGTCGT AGCAGGAGCG CTGGGCGTGG   1260
TGCCTAAAAA TGTCTTTCAG TAGCAAGCTG ATTGCCAGGG GCAGGCCCTT GGTGTAAGTG   1320
TTTACAAAGC GGTTAAGCTG GGATGGGTGC ATACGTGGGG ATATGAGATG CATCTTGGAC   1380
TGTATTTTTA GGTTGGCTAT GTTCCCAGCC ATATCCCTCC GGGGATTCAT GTTGTGCAGA   1440
ACCACCAGCA CAGTGTATCC GGTGCACTTG GGAAATTTGT CATGTAGCTT AGAAGGAAAT   1500
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GCGTGGAAGA ACTTGGAGAC GCCCTTGTGA CCTCCAAGAT TTTCCATGCA TTCGTCCATA 1560
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TCATAGTTGT GTTCCAGGAT GAGATCGTCA TAGGCCATTT TTACAAAGCG CGGGCGGAGG 1680
GTGCCAGACT GCGGTATAAT GGTTCCATCC GGCCCAGGGG CGTAGTTACC CTCACAGATT 1740
TGCATTTCCC ACGCTTTGAG TTCAGATGGG GGGATCATGT CTACCTGCGG GGCGATGAAG 1800

Claim

1. A nucleotide sequence which contains elements of an adenovirus genome and a heterologous gene of mammalian origin that is under the control therein of a eucaryotic transcriptional promoter, said sequence being capable of functioning as a vector from which expression of said heterologous gene can be directed when said vector is placed in a cell of an individual, wherein said nucleotide sequence is further characterized by:

(a) absence therefrom of a first element of the adenovirus genome that is essential to replication or packaging of adenovirus in a host mammalian cell; and

(b) placement in said nucleotide sequence at, or directly adjacent to, the location in said nucleotide sequence otherwise occupied by said first element, of a second element of adenovirus genome that is itself essential to the replication or packaging of adenovirus in a host mammalian cell.

2. A nucleotide sequence according to Claim 1 wherein said first element consists essentially of the E1a-E1b region of adenovirus genome and said second element thereof is selected from the group consisting of the E4 region, E2A, the gene encoding terminal protein, the fiber encoding gene (L5), ORF6, and adenovirus structural proteins.

3. A nucleotide sequence which contains elements of an adenovirus genome and a heterologous gene of mammalian origin that is under the control therein of a eucaryotic transcriptional promoter, said sequence being capable of functioning as a vector

from which expression of said heterologous gene can be directed when said vector is placed in a cell of an individual, wherein said nucleotide sequence is further characterized by:

(a) the absence therefrom of the E1a-E1b region of the adenovirus genome; and

(b) placement of a stuffer sequence in said nucleotide sequence in a region other than that of the heterologous gene of mammalian origin, said vector being further characterized in that legitimate recombination of said sequence with an element that is present in a helper cell used to replicate or package said sequence, or with an element that is present in a cell of an individual, and having homology with said E1a-E1b region, leads to the production of a lengthened nucleotide sequence that is substantially less efficient than said unmodified nucleotide sequence at being packaged in said helper cell or in a cell of said individual.

4. A nucleotide sequence which contains elements of an adenovirus genome, including the gene for adenoviral protein IX, and a heterologous gene of mammalian origin that is under the control therein of a eucaryotic transcriptional promoter, said sequence being capable of functioning as a vector from which expression of said heterologous gene can be directed when said vector is placed in a cell of an individual, wherein said nucleotide sequence is further characterized by:

(a) absence therefrom of the E1a-E1b region of the adenovirus genome; and

(b) repositioning of the gene that encodes protein IX to a location that deviates from its

normal location in the wild-type adenovirus genome.

5. The nucleotide sequence of Claim 4, which is Ad2/CFTR-8.

6. A method for minimizing exposure of an individual undergoing gene therapy that involves a virus vector to deliver a heterologous gene to replication-competent virus comprising the step of treating said individual with a gene therapy composition that itself comprises:

(1) a pharmaceutically acceptable carrier, and

(2) a vector in the form of a nucleotide sequence that includes elements of a viral genome and a heterologous gene of mammalian origin under the control therein of a eucaryotic transcriptional promoter, such that expression of said heterologous gene can be directed when said vector is placed in a cell of a patient, said vector being further characterized by

(a) absence therefrom of a first element of viral genome that is essential to the replication or packaging of said virus in a host mammalian cell, and

(b) placement in said nucleotide sequence at, or directly adjacent to, the position in said nucleotide sequence otherwise occupied by said first element, of a second element of viral genome that is itself essential to the replication or packaging of said virus in a host mammalian cell.

7. A method of providing a vector for use in gene therapy wherein said vector comprises elements of adenoviral genome and has a substantially reduced tendency to generate replication-competent adenovirus through a legitimate recombinational event with an adenoviral element that is present in a helper cell used to replicate and package said vector, said method comprising:

(1) providing said vector as a nucleotide sequence according to Claim 1, and

(2) replicating and packaging said vector in helper cells that provide expression of said first element of adenoviral genome in trans, and wherein said sequence tends to eliminate said second essential element thereof as a consequence of recombination with a copy of said first element provided from said helper cell.

8. A nucleotide sequence which contains elements of an adenovirus genome, and a heterologous gene of mammalian origin that is under the control therein of a eucaryotic transcriptional promoter, said sequence being capable of functioning as a vector from which expression of said heterologous gene can be directed when said vector is placed in a cell of an individual, wherein said nucleotide sequence is further characterized by:

(a) absence therefrom of the E1a-E1b region of the adenovirus genome; and

(b) absence therefrom of the protein IX region of the adenovirus genome; and

(c) a sequence size that does not exceed about 90% of the length of the adenovirus genome.

9. The nucleotide sequence of Claim 8, which is Ad2/CFTR-7.

10. A nucleotide sequence which contains elements of an adenovirus genome, and a heterologous gene of mammalian origin that is under the control therein of a eucaryotic transcriptional promoter, said sequence being capable of functioning as a vector from which expression of said heterologous gene can be directed when said vector is placed in a cell of an individual, wherein said sequence is further characterized by:

(a) absence therefrom of the E1a-E1b region of the adenovirus genome; and

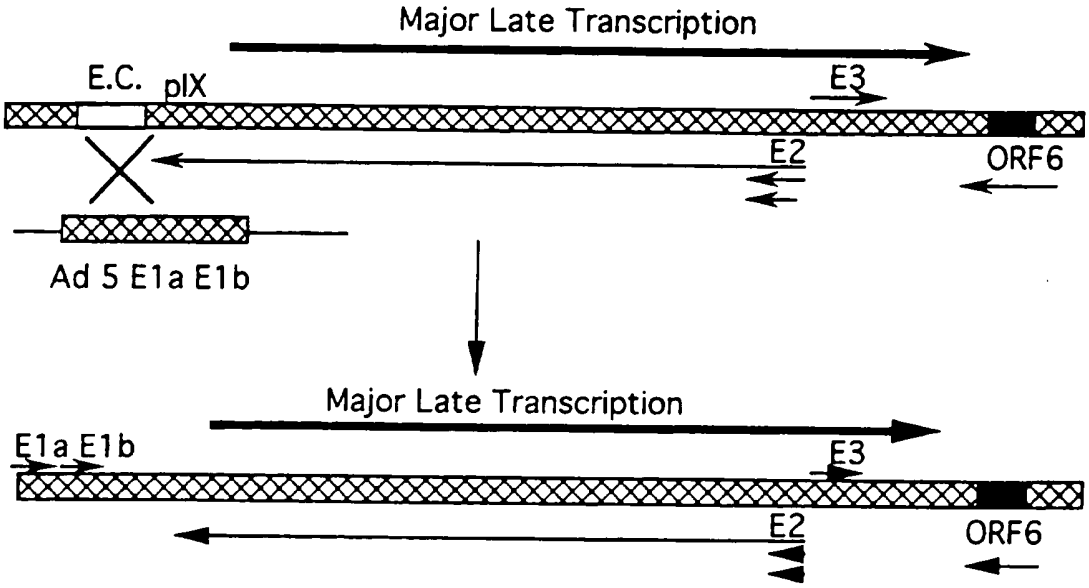
(b) absence therefrom of the E4 region of the adenovirus genome except for the ORF6 region.

11. The nucleotide sequence of Claim 10 in which the eucaryotic transcriptional promoter is selected from the group consisting of the cytomegalovirus, phosphoglycerate kinase, and adenovirus major late protein promoters.

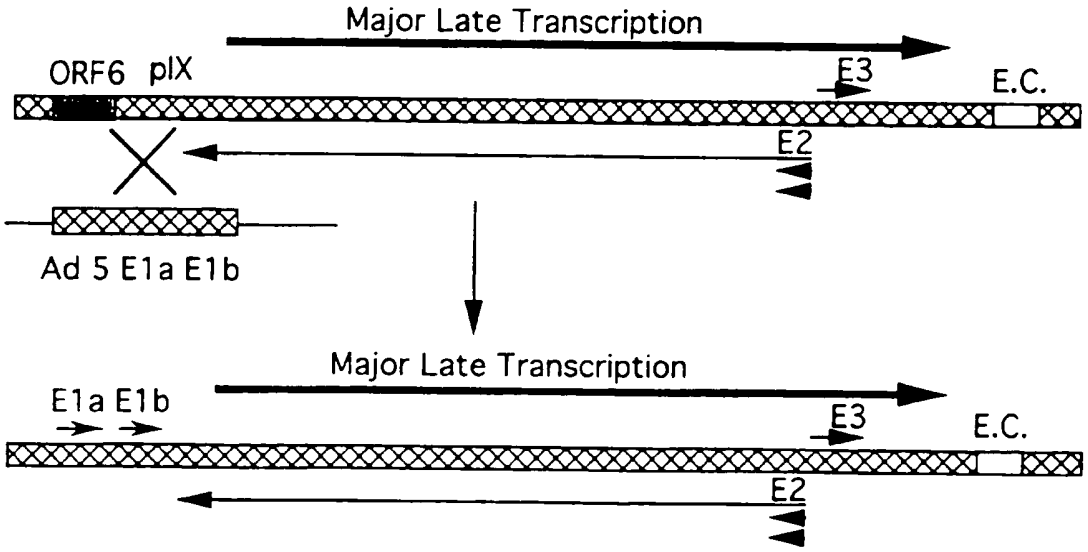
12. The nucleotide sequence of Claim 10, which is Ad2/CFTR-5.

13. The nucleotide sequence of Claim 10, which is Ad2/CFTR-4.

Current vector structure

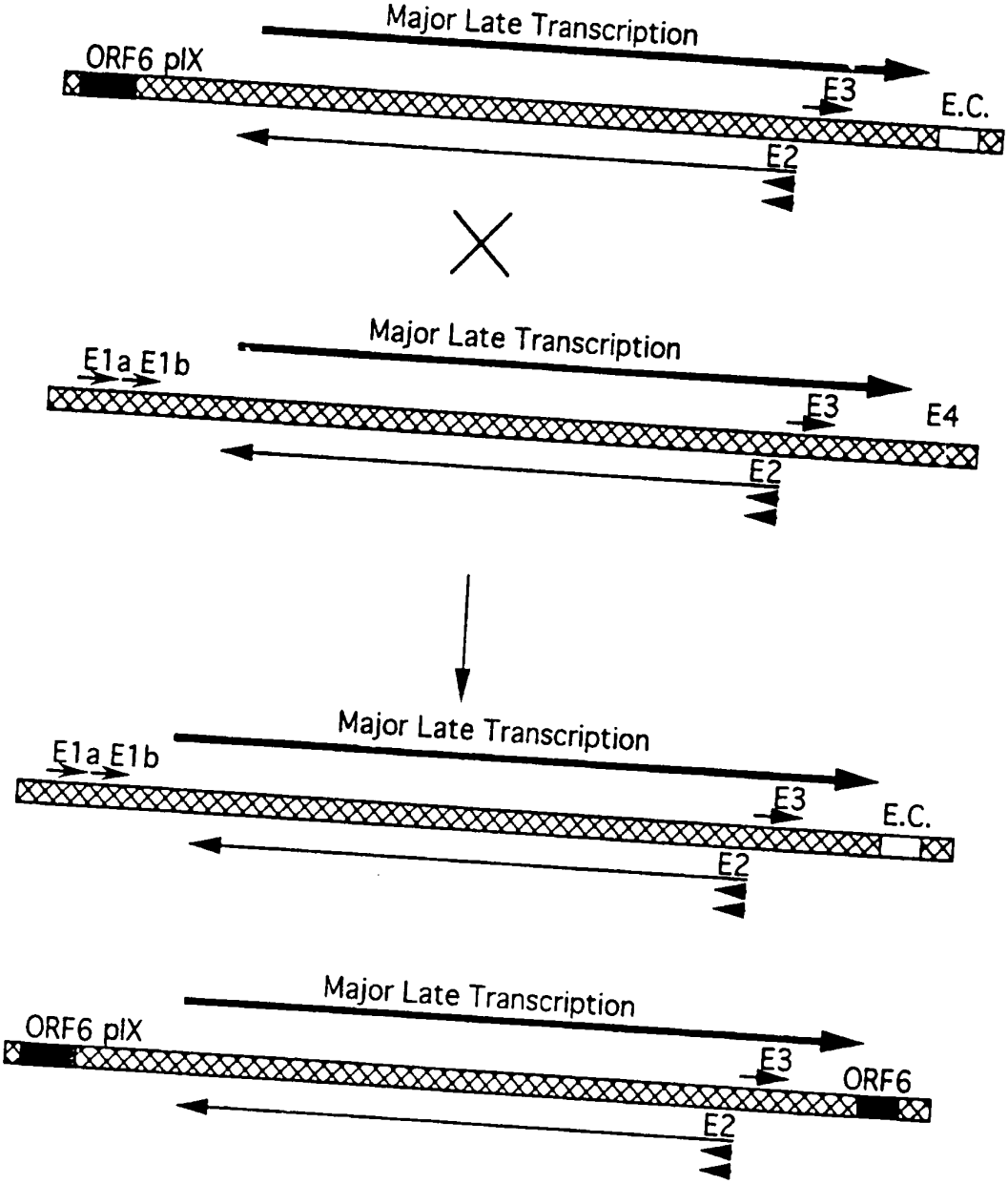


New structure



Strategy for the Prevention of RCA Generation in 293 cells

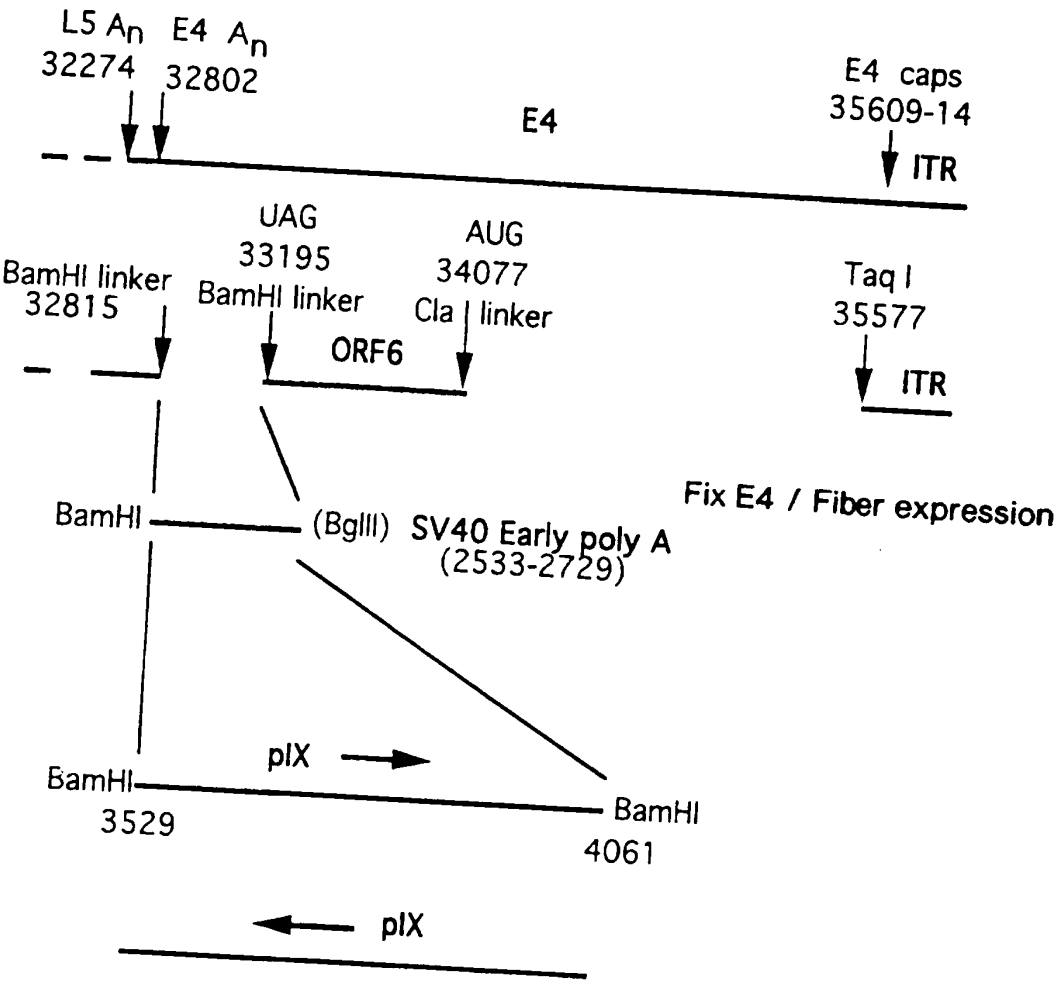
FIG. 1



Result of recombination between vectors with new design and wild type virus

E.C.= expresson cassette containing transgene of interest

FIG. 2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32274
E4 An
32802
E4
E4 caps
35609-14
ITR
UAG
33195
BamHI linker
AUG
34077
Cla I linker
BamHI linker
32815
ORF6
Taq I
35577
ITR
Fix E4 / Fiber expression
BamHI
(BglII)
SV40 Early poly A
(2533-2729)
pIX
BamHI
3529
BamHI
4061
pIX
New Poly A E4 / pIX

FIG. 3

Ad2.Seq: (Top Strand) x Ad5.Seq (Bottom Strand)

```
   1 CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAG  49
     ||||||||||||||||||||||||||||||||||||||||||||||||||
   1 CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAG  50

  50 GGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACG  99
     ||||||||||||||||||||||||||||||||||||||||||||||||||
  51 GGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACG 100

 100 TAGTAGTGTGGCGGAAGTGTGATGTTGCAAGTGTGGCGGAACACATGTAA 149
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 101 TAGTAGTGTGGCGGAAGTGTGATGTTGCAAGTGTGGCGGAACACATGTAA 150

 150 GCGCCGGATGTGGTAAAAGTGACGTTTTTGGTGTGCGCCGGTGTATACGG 199
     ||| |||||||||| ||||||||||||||||||||||||||||| || |
 151 GCGACGGATGTGGCAAAAGTGACGTTTTTGGTGTGCGCCGGTGTACACAG 200

 200 GAAGTGACAATTTTCGCGCGGTTTTAGGCGGATGTTGTAGTAAATTTGGG 249
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 201 GAAGTGACAATTTTCGCGCGGTTTTAGGCGGATGTTGTAGTAAATTTGGG 250

 250 CGTAACCAAGTAATATTTGGCCATTTTCGCGGGAAAACTGAATAAGAGGA 299
     ||||||| ||||| ||||||||||||||||||||||||||||||||||||
 251 CGTAACCGAGTAAGATTTGGCCATTTTCGCGGGAAAACTGAATAAGAGGA 300

 300 AGTGAAATCTGAATAATTCTGTGTTACTCATAGCGCGTAATATTTGTCTA 349
     |||||||||||||||||| ||||||||||||||||||||||||||||||
 301 AGTGAAATCTGAATAATTTTGTGTTACTCATAGCGCGTAATATTTGTCTA 350

 350 GGGCCGCGGGGACTTTGACCGTTTACGTGGAGACTCGCCCAGGTGTTTTT 399
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 351 GGGCCGCGGGGACTTTGACCGTTTACGTGGAGACTCGCCCAGGTGTTTTT 400

 400 CTCAGGTGTTTTCCGCGTTCCGGGTCAAAGTTGGCGTTTTATTATTATAG 449
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 401 CTCAGGTGTTTTCCGCGTTCCGGGTCAAAGTTGGCGTTTTATTATTATAG 450

 450 TCAGCTGACGCGCAGTGTATTTATACCCGGTGAGTTCCTCAAGAGGCCAC 499
     |||||||||| | ||||||||||||||||||||||||||||||||||||
 451 TCAGCTGACGTGTAGTGTATTTATACCCGGTGAGTTCCTCAAGAGGCCAC 500

 500 TCTTGAGTGCCAGCGAGTAGAGTTTTCTCCTCCGAGCCGCTCCGACACCG 549
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 501 TCTTGAGTGCCAGCGAGTAGAGTTTTCTCCTCCGAGCCGCTCCGACACCG 550

 550 GGACTGAAAATGAGACATATTATCTGCCACGGAGGTGTTATTACCGAAGA 599
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 551 GGACTGAAAATGAGACATATTATCTGCCACGGAGGTGTTATTACCGAAGA 600

     600.....3041
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FIG. 4A

```
3042 CATAACATGGTGTGTGGCAACTGCGAGGACAGGGCCTCTCAGATGCTGAC 3091
     ||||||||||| ||||||||||||||||||||||||||||||||||||||
3048 CATAACATGGTATGTGGCAACTGCGAGGACAGGGCCTCTCAGATGCTGAC 3097

3092 CTGCTCGGACGGCAACTGTCACTTGCTGAAGACCATTCACGTAGCCAGCC 3141
     |||||||||||||||||||| ||||||||||||||||||||||||||||
3098 CTGCTCGGACGGCAACTGTCACCTGCTGAAGACCATTCACGTAGCCAGCC 3147

3142 ACTCTCGCAAGGCCTGGCCAGTGTTTGAGCACAACATACTGACCCGCTGT 3191
     |||||||||||||||||||||||||||||| ||||||||||||||||||
3148 ACTCTCGCAAGGCCTGGCCAGTGTTTGAGCATAACATACTGACCCGCTGT 3197

3192 TCCTTGCATTTGGGTAACAGGAGGGGGGTGTTCCTACCTTACCAATGCAA 3241
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3198 TCCTTGCATTTGGGTAACAGGAGGGGGGTGTTCCTACCTTACCAATGCAA 3247

3242 TTTGAGTCACACTAAGATATTGCTTGAGCCCGAGAGCATGTCCAAGGTGA 3291
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3248 TTTGAGTCACACTAAGATATTGCTTGAGCCCGAGAGCATGTCCAAGGTGA 3297

3292 ACCTGAACGGGGTGTTTGACATGACCATGAAGATCTGGAAGGTGCTGAGG 3341
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3298 ACCTGAACGGGGTGTTTGACATGACCATGAAGATCTGGAAGGTGCTGAGG 3347

3342 TACGATGAGACCCGCACCAGGTGCAGACCCTGCGAGTGTGGCGGTAAACA 3391
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3348 TACGATGAGACCCGCACCAGGTGCAGACCCTGCGAGTGTGGCGGTAAACA 3397

3392 TATTAGGAACCAGCCTGTGATGCTGGATGTGACCGAGGAGCTGAGGCCCG 3441
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3398 TATTAGGAACCAGCCTGTGATGCTGGATGTGACCGAGGAGCTGAGGCCCG 3447

3442 ATCACTTGGTGCTGGCCTGCACCCGCGCTGAGTTTGGCTCTAGCGATGAA 3491
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3448 ATCACTTGGTGCTGGCCTGCACCCGCGCTGAGTTTGGCTCTAGCGATGAA 3497

3492 GATACAGATTGAGGTACTGAAATGTGTGGGCGTGGCTTAAGGGTGGGAAA 3541
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3498 GATACAGATTGAGGTACTGAAATGTGTGGGCGTGGCTTAAGGGTGGGAAA 3547

3542 GAATATATAAGGTGGGGGTCTCATGTAGTTTTGTATCTGTTTTGCAGCA. 3590
     |||||||||||||||||||| ||||||||||||||||||||||||||
3548 GAATATATAAGGTGGGGGTCTTATGTAGTTTTGTATCTGTTTTGCAGCAG 3597

3591 ..GCCGCCGCCATGAGCGCCAACTCGTTTGATGGAAGCATTGTGAGCTCA 3638
       |||||||||||||| ||||||||||||||||||||||||||||||||
3598 CCGCCGCCGCCATGAGCACCAACTCGTTTGATGGAAGCATTGTGAGCTCA 3647

3639 TATTTGACAACGCGCATGCCCCCATGGGCCGGGGGTGCGTCAGAATGTGAT 3688
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3648 TATTTGACAACGCGCATGCCCCCATGGGCCGGGGGTGCGTCAGAATGTGAT 3697

3689 GGGCTCCAGCATTGATGGTCGCCCCGTCCTGCCCGCAAACTCTACTACCT 3738
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3698 GGGCTCCAGCATTGATGGTCGCCCCGTCCTGCCCGCAAACTCTACTACCT 3747
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FIG. 4B

```
3739 TGACCTACGAGACCGTGTCTGGAACGCCGTTGGAGACTGCAGCCTCCGCC 3788
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3748 TGACCTACGAGACCGTGTCTGGAACGCCGTTGGAGACTGCAGCCTCCGCC 3797

3789 GCCGCTTCAGCCGCTGCAGCCACCGCCCGCGGGATTGTGACTGACTTTGC 3838
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3798 GCCGCTTCAGCCGCTGCAGCCACCGCCCGCGGGATTGTGACTGACTTTGC 3847

3839 TTTCCTGAGCCCGCTTGCAAGCAGTGCAGCTTCCCGTTCATCCGCCCGCG 3888
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3848 TTTCCTGAGCCCGCTTGCAAGCAGTGCAGCTTCCCGTTCATCCGCCCGCG 3897

3889 ATGACAAGTTGACGGCTCTTTTGGCACAATTGGATTCTTTGACCCGGGAA 3938
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3898 ATGACAAGTTGACGGCTCTTTTGGCACAATTGGATTCTTTGACCCGGGAA 3947

3939 CTTAATGTCGTTTCTCAGCAGCTGTTGGATCTGCGCCAGCAGGTTTCTGC 3988
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3948 CTTAATGTCGTTTCTCAGCAGCTGTTGGATCTGCGCCAGCAGGTTTCTGC 3997

3989 CCTGAAGGCTTCCTCCCCTCCCAATGCGGTTTAAAACATAAAT.AAAAAC 4037
     ||||||||||||||||||||||||||||||||||||||||||| ||||||
3998 CCTGAAGGCTTCCTCCCCTCCCAATGCGGTTTAAAACATAAATAAAAAAC 4047

4038 CAGACTCTGTTTGGATTTTGATCAAGCAAGTGTCTTGCTGTCTTTATTTA 4087
     |||||||||||||||||| ||||||||||||||||||||||||||||||
4048 CAGACTCTGTTTGGATTTGGATCAAGCAAGTGTCTTGCTGTCTTTATTTA 4097

4088 GGGGTTTTGCGCGCGCGGTAGGCCCGGGACCAGCGGTCTCGGTCGTTGAG 4137
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4098 GGGGTTTTGCGCGCGCGGTAGGCCCGGGACCAGCGGTCTCGGTCGTTGAG 4147

4138 GGTCCTGTGTATTTTTTCCAGGACGTGGTAAAGGTGACTCTGGATGTTCA 4187
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4148 GGTCCTGTGTATTTTTTCCAGGACGTGGTAAAGGTGACTCTGGATGTTCA 4197

4188 GATACATGGGCATAAGCCCGTCTCTGGGGTGGAGGTAGCACCACTGCAGA 4237
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4198 GATACATGGGCATAAGCCCGTCTCTGGGGTGGAGGTAGCACCACTGCAGA 4247

4238 GCTTCATGCTGCGGGGTGGTGTTGTAGATGATCCAGTCGTAGCAGGAGCG 4287
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4248 GCTTCATGCTGCGGGGTGGTGTTGTAGATGATCCAGTCGTAGCAGGAGCG 4297

4288 CTGGGCGTGGTGCCTAAAAATGTCTTTCAGTAGCAAGCTGATTGCCAGGG 4337
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4298 CTGGGCGTGGTGCCTAAAAATGTCTTTCAGTAGCAAGCTGATTGCCAGGG 4347

4338 GCAGGCCCTTGGTGTAAGTGTTTACAAAGCGGTTAAGCTGGGATGGGTGC 4387
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4348 GCAGGCCCTTGGTGTAAGTGTTTACAAAGCGGTTAAGCTGGGATGGGTGC 4397

4388 ATACGTGGGGATATGAGATGCATCTTGGACTGTATTTTTAGGTTGGCTAT 4437
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4398 ATACGTGGGGATATGAGATGCATCTTGGACTGTATTTTTAGGTTGGCTAT 4447
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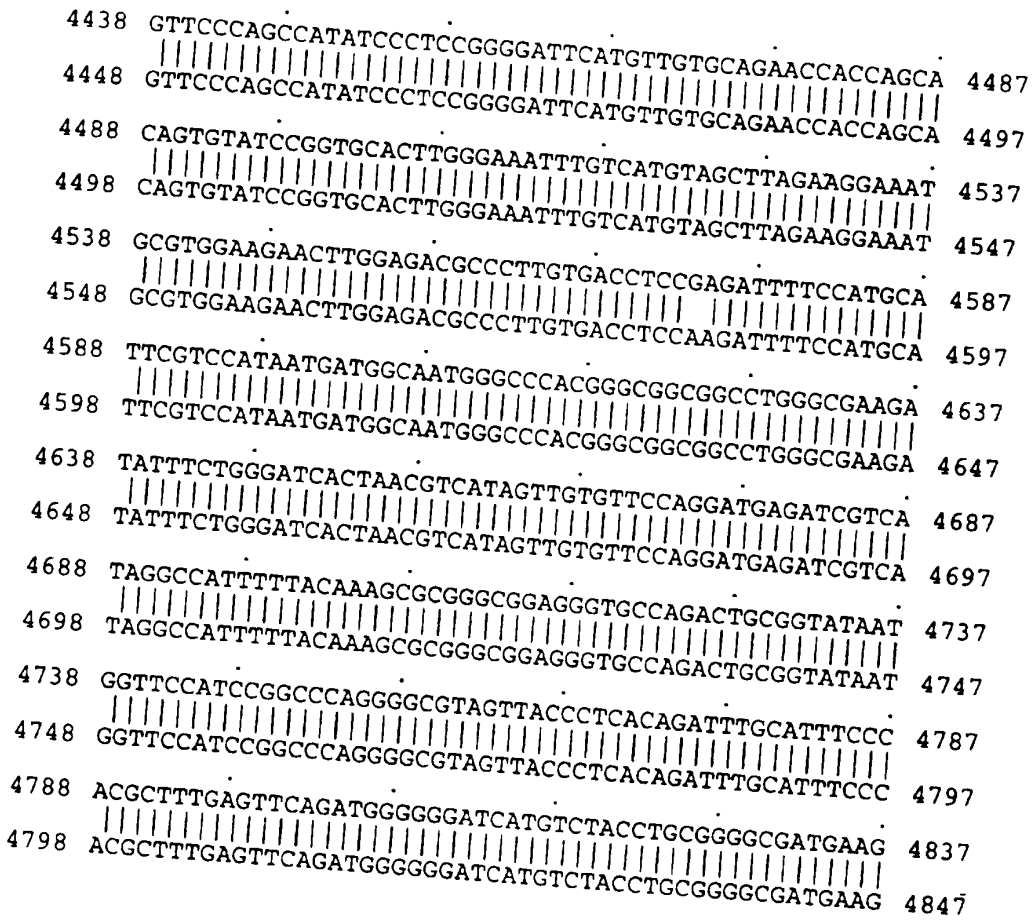
FIG. 4C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4448 GTTCCCAGCCATATCCCTCCGGGGATTCATGTTGTGCAGAACCACCAGCA
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4498 CAGTGTATCCGGTGCACTTGGGAAATTTGTCATGTAGCTTAGAAGGAAAT
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4548 GCGTGGAAGAACTTGGAGACGCCCTTGTGACCTCCAAGATTTTCCATGCA
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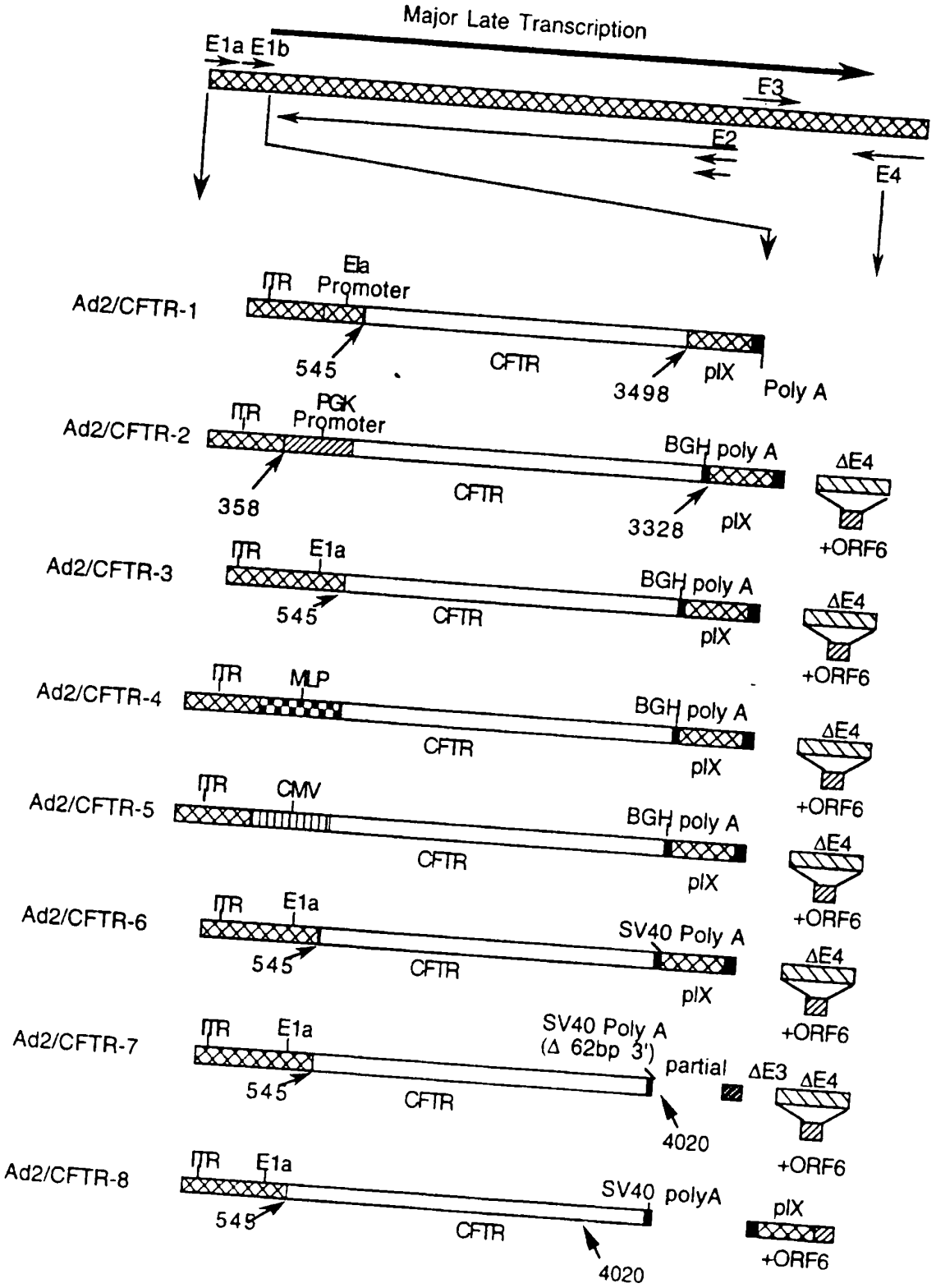
4598 TTCGTCCATAATGATGGCAATGGGCCCACGGGCGGCGGCCTGGGCGAAGA
4638 TATTTCTGGGATCACTAACGTCATAGTTGTGTTCCAGGATGAGATCGTCA 4687
4648 TATTTCTGGGATCACTAACGTCATAGTTGTGTTCCAGGATGAGATCGTCA
4688 TAGGCCATTTTTACAAAGCGCGGGCGGAGGGTGCCAGACTGCGGTATAAT 4737
4698 TAGGCCATTTTTACAAAGCGCGGGCGGAGGGTGCCAGACTGCGGTATAAT
4738 GGTTCCATCCGGCCCAGGGGCGTAGTTACCCTCACAGATTTGCATTTCCC 4787
4748 GGTTCCATCCGGCCCAGGGGCGTAGTTACCCTCACAGATTTGCATTTCCC
4788 ACGCTTTGAGTTCAGATGGGGGGATCATGTCTACCTGCGGGGCGATGAAG 4837
4798 ACGCTTTGAGTTCAGATGGGGGGATCATGTCTACCTGCGGGGCGATGAAG 4847

FIG. 4D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E3
E2
E4
Ad2/CFTR-1
ITR
E1a Promoter
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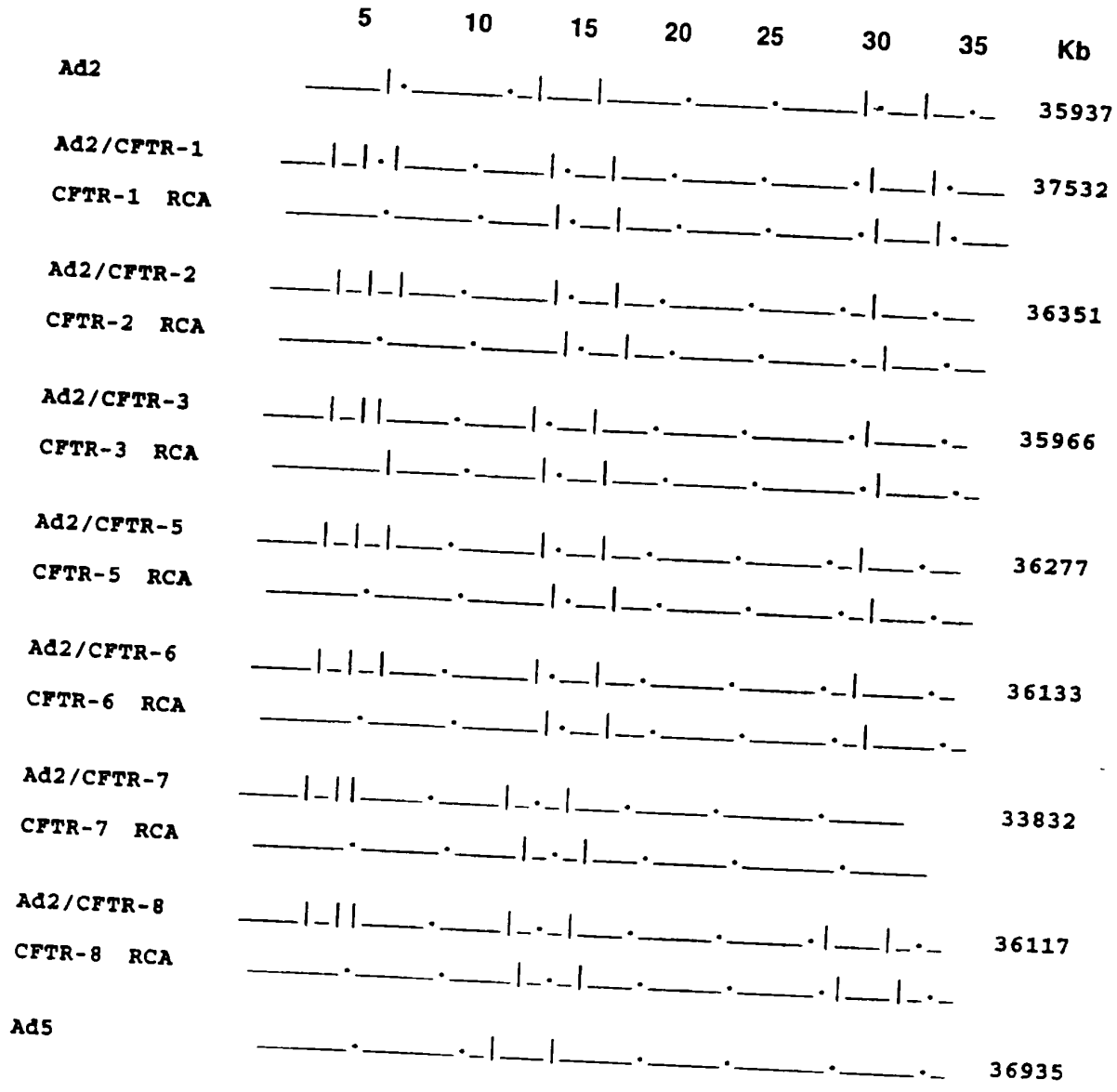
CFTR
3498
pIX
Poly A
Ad2/CFTR-2
ITR
PGK Promoter
358
CFTR
BGH poly A
3328
pIX
ΔE4
+ORF6
Ad2/CFTR-3
ITR
E1a
545
CFTR
BGH poly A
pIX
ΔE4
+ORF6
Ad2/CFTR-4
ITR
MLP
CFTR
BGH poly A
pIX
ΔE4
+ORF6
Ad2/CFTR-5
ITR
CMV
CFTR
BGH poly A
pIX
ΔE4
+ORF6
Ad2/CFTR-6
ITR
E1a
545
CFTR
SV40 Poly A
pIX
ΔE4
+ORF6
Ad2/CFTR-7
ITR
E1a
545
CFTR
SV40 Poly A
(Δ 62bp 3')
4020
partial
ΔE3
ΔE4
+ORF6
Ad2/CFTR-8
ITR
E1a
545
CFTR
SV40 polyA
4020
pIX
+ORF6

FIG. 5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10
15
20
25
30
35
Kb
Ad2
35937
Ad2/CFTR-1
CFTR-1 RCA
37532
Ad2/CFTR-2
CFTR-2 RCA
36351
Ad2/CFTR-3
CFTR-3 RCA
35966
Ad2/CFTR-5
CFTR-5 RCA
36277
Ad2/CFTR-6
CFTR-6 RCA
36133
Ad2/CFTR-7
CFTR-7 RCA
33832
Ad2/CFTR-8
CFTR-8 RCA
36117
Ad5
36935

FIG. 6

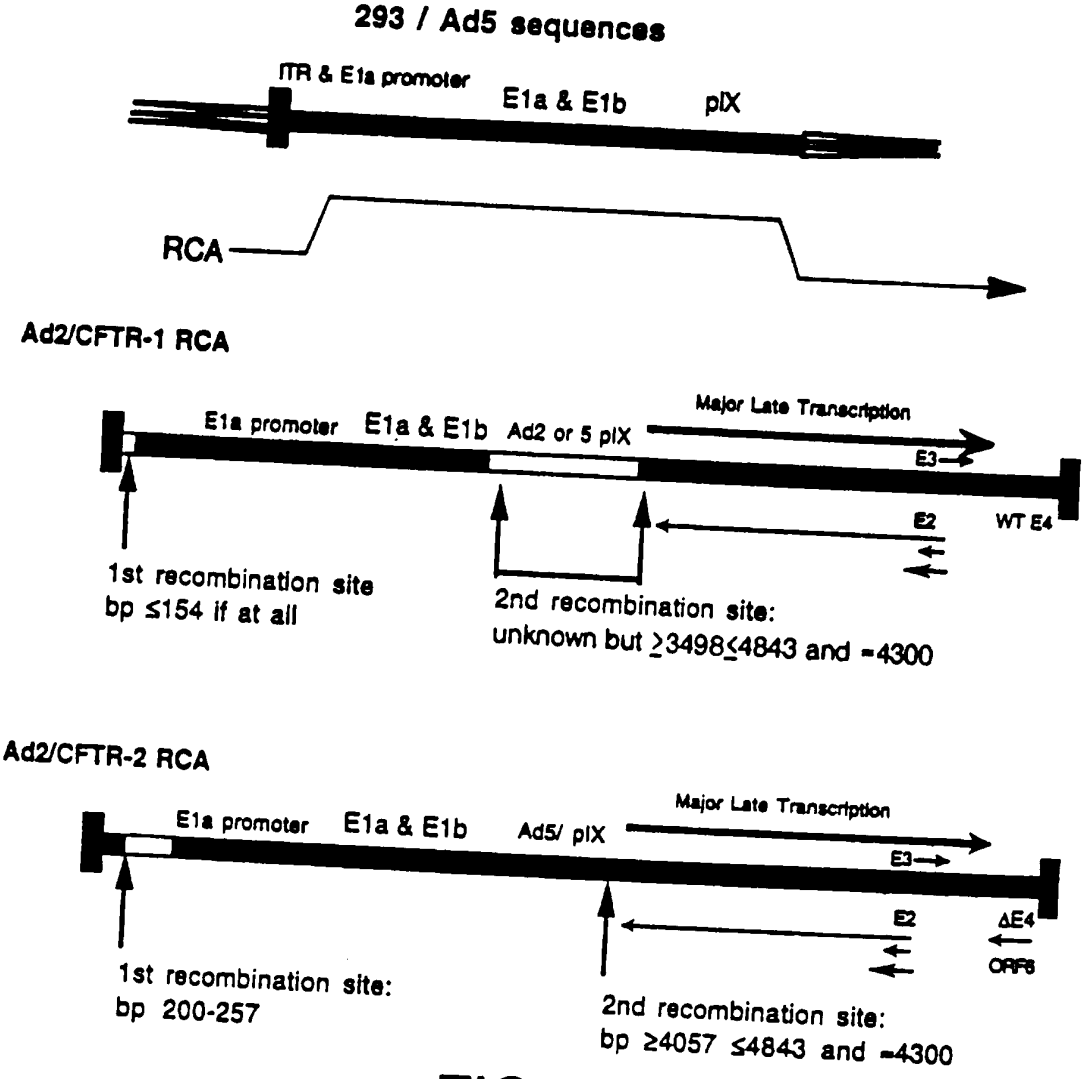
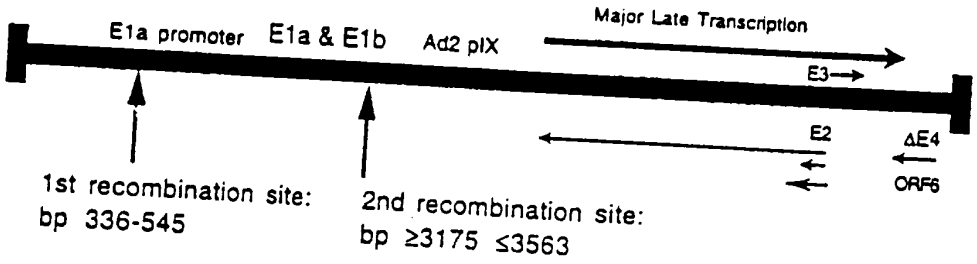
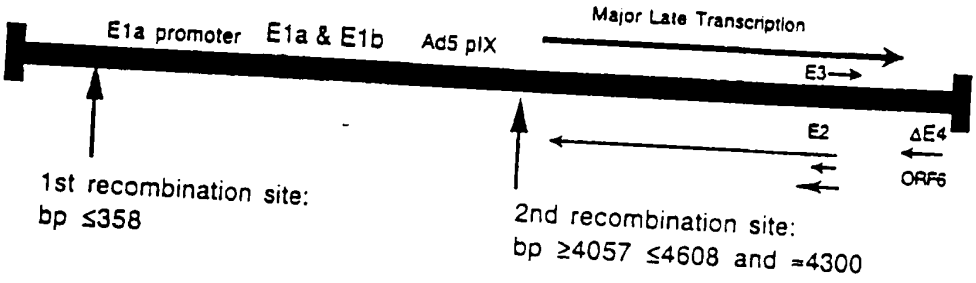

293 / Ad5 sequences
ITR & E1a promoter
E1a & E1b
pIX
RCA
Ad2/CFTR-1 RCA
E1a promoter
E1a & E1b
Ad2 or 5 pIX
Major Late Transcription
E3
E2
WT E4
1st recombination site
bp ≤154 if at all
2nd recombination site:
unknown but ≥3498≤4843 and ≈4300
Ad2/CFTR-2 RCA
E1a promoter
E1a & E1b
Ad5/ pIX
Major Late Transcription
E3
E2
ΔE4
ORF6
1st recombination site:
bp 200-257
2nd recombination site:
bp ≥4057 ≤4843 and ≈4300

FIG. 7A

Ad2/CFTR-3 RCA



Ad2/CFTR-5 RCA



Ad2/CFTR-6 RCA

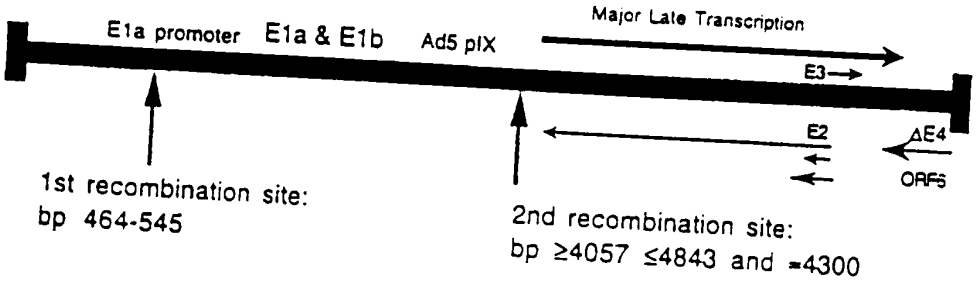


FIG. 7B

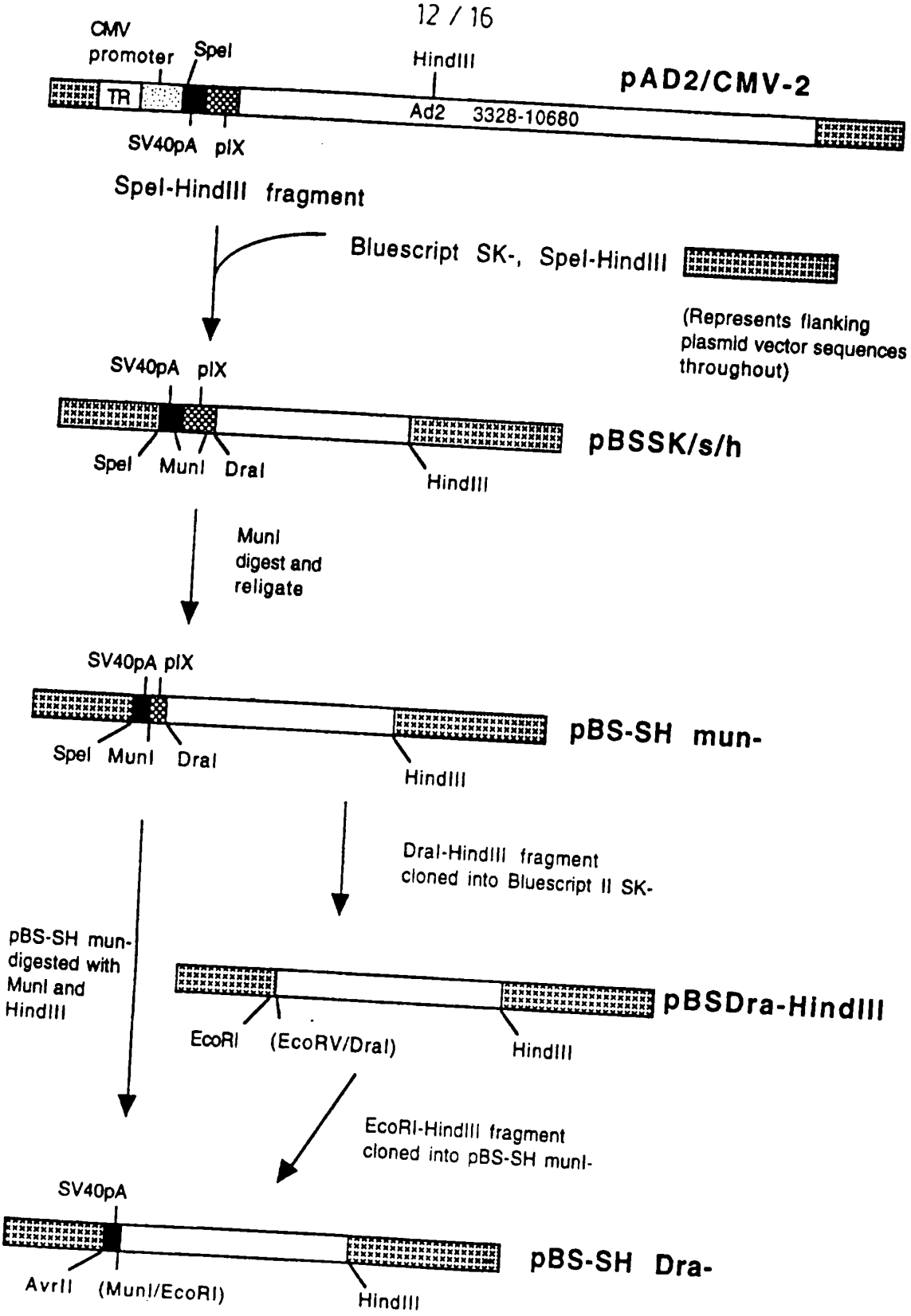

CMV promoter
SpeI
HindIII
pAD2/CMV-2
TR
Ad2 3328-10680
SV40pA
pIX
SpeI-HindIII fragment
Bluescript SK-, SpeI-HindIII
(Represents flanking plasmid vector sequences throughout)
SV40pA pIX
SpeI MunI DraI
HindIII
pBSSK/s/h
MunI digest and religate
SV40pA pIX
SpeI MunI DraI
HindIII
pBS-SH mun-
DraI-HindIII fragment cloned into Bluescript II SK-
pBS-SH mun- digested with MunI and HindIII
EcoRI
(EcoRV/DraI)
HindIII
pBSDra-HindIII
EcoRI-HindIII fragment cloned into pBS-SH munI-
SV40pA
AvrII
(MunI/EcoRI)
HindIII
pBS-SH Dra-

FIG. 8A

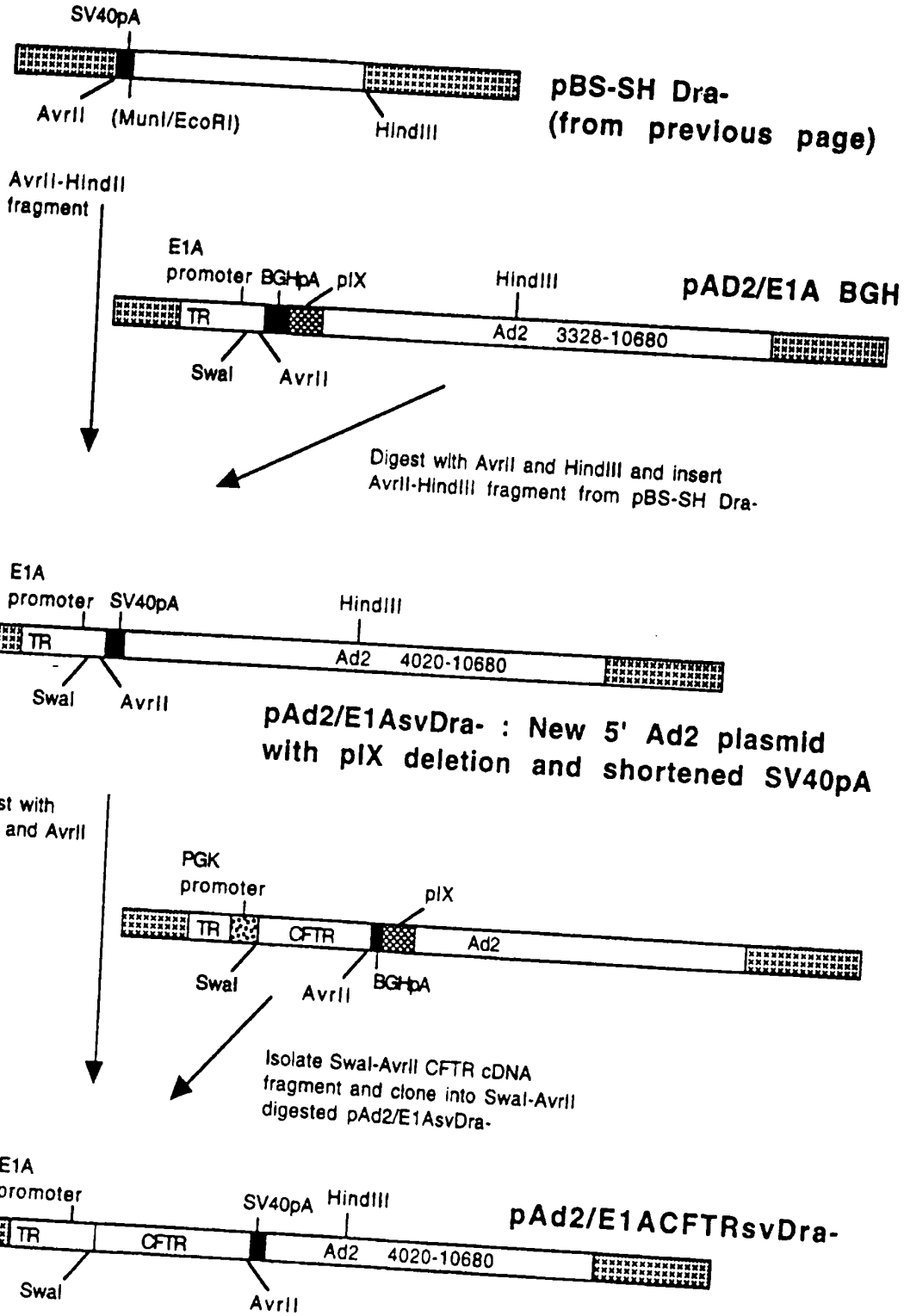

SV40pA
AvrII
(MunI/EcoRI)
HindIII
pBS-SH Dra-
(from previous page)
AvrII-HindII
fragment
E1A
promoter
BGHpA
pIX
HindIII
pAD2/E1A BGH
TR
Ad2 3328-10680
SwaI
AvrII
Digest with AvrII and HindIII and insert
AvrII-HindIII fragment from pBS-SH Dra-
E1A
promoter
SV40pA
HindIII
TR
Ad2 4020-10680
SwaI
AvrII
pAd2/E1AsvDra- : New 5' Ad2 plasmid
with pIX deletion and shortened SV40pA
Digest with
SwaI and AvrII
PGK
promoter
pIX
TR
CFTR
Ad2
SwaI
AvrII
BGHpA
Isolate SwaI-AvrII CFTR cDNA
fragment and clone into SwaI-AvrII
digested pAd2/E1AsvDra-
E1A
promoter
SV40pA
HindIII
pAd2/E1ACFTRsvDra-
TR
CFTR
Ad2 4020-10680
SwaI
AvrII

FIG. 8B

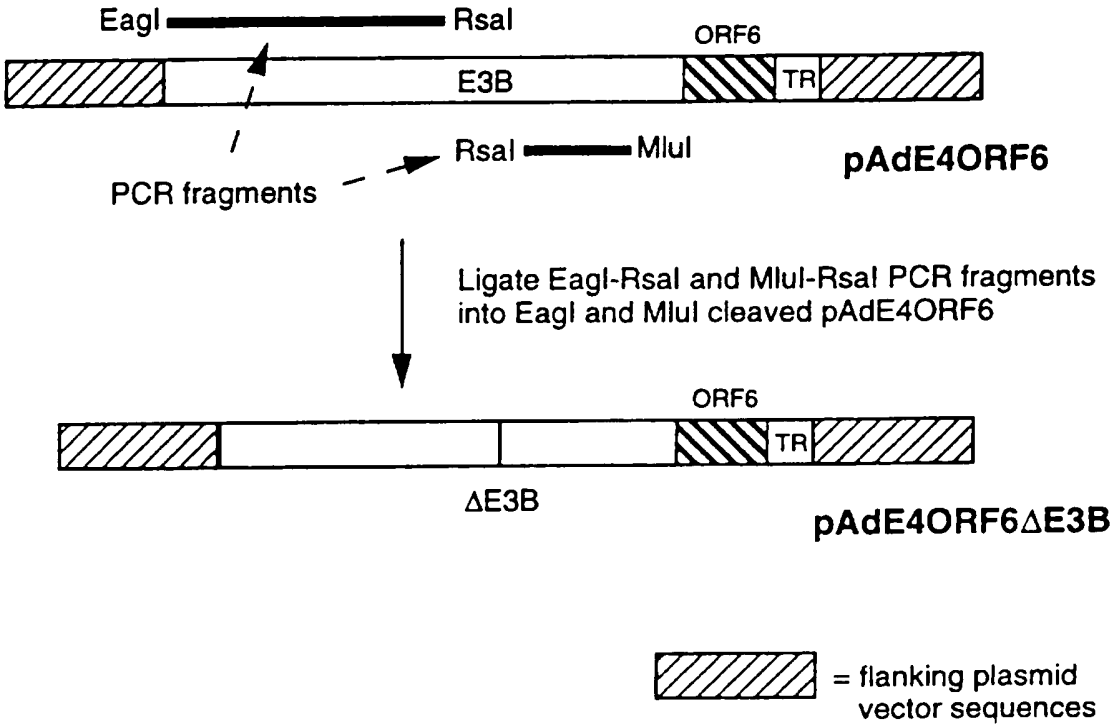

EagI
RsaI
ORF6
E3B
TR
RsaI
MluI
pAdE4ORF6
PCR fragments
Ligate EagI-RsaI and MluI-RsaI PCR fragments
into EagI and MluI cleaved pAdE4ORF6
ORF6
TR
ΔE3B
pAdE4ORF6ΔE3B
= flanking plasmid
vector sequences

FIG. 9

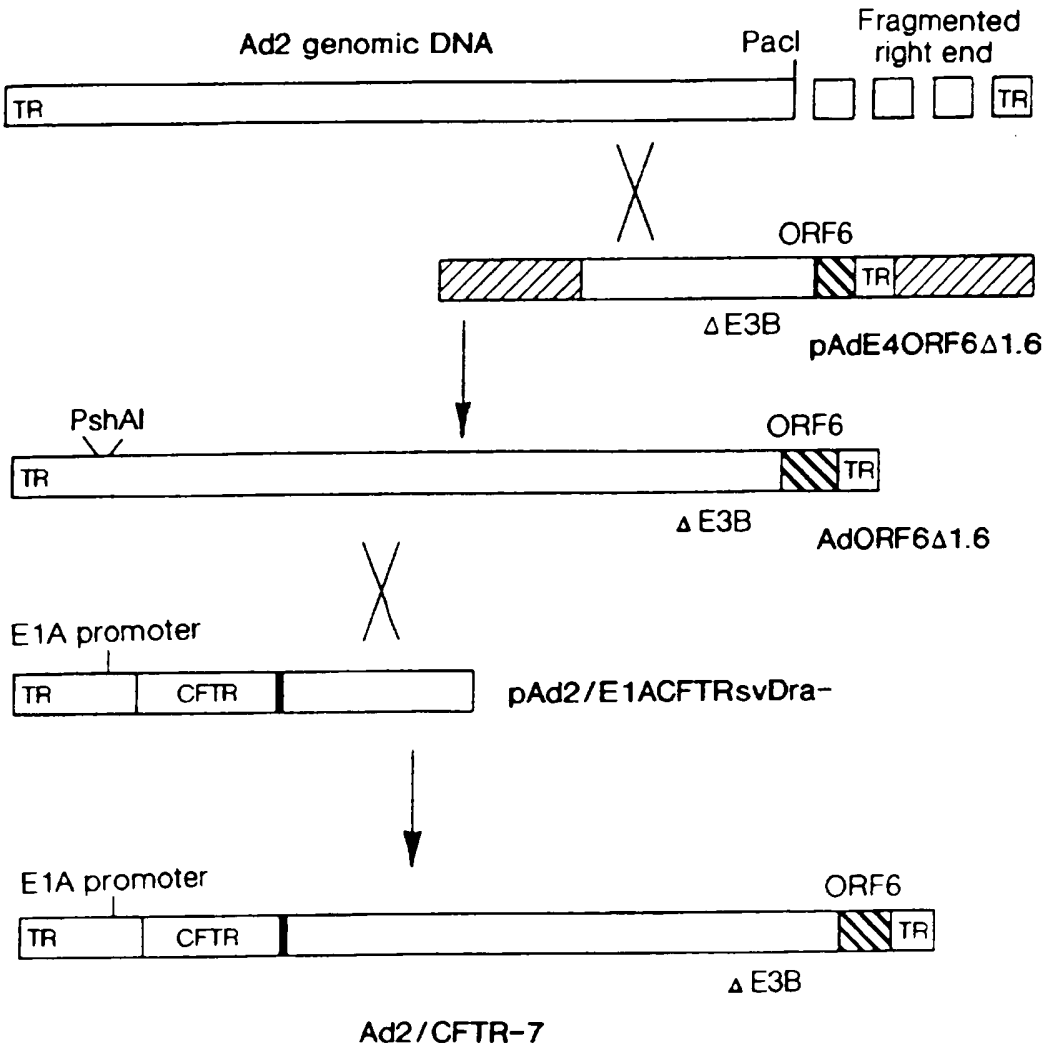

Ad2 genomic DNA
PacI
Fragmented right end
TR
TR
ORF6
TR
ΔE3B
pAdE4ORF6Δ1.6
PshAI
ORF6
TR
TR
ΔE3B
AdORF6Δ1.6
E1A promoter
TR
CFTR
pAd2/E1ACFTRsvDra-
E1A promoter
ORF6
TR
CFTR
TR
ΔE3B
Ad2/CFTR-7

FIG. 10

(* The virus from this passage # is not the same as the virus from the original passage since a different aliquot from the previous passage was used to make a 1roller bottle CsCl gradient prep for the higher dose RCA tests. However, the level of RCA in this passage # should be the same as in the original passage.)

FIG. 11

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 96/03818

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N15/12 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUMAN GENE THERAPY, vol. 6, no. 2, February 1995, MARY ANN LIEBERT, INC. PUBLISHERS, NEW YORK, US, pages 205-218, XP000575815 M.J. WELSH ET AL.: "Adenovirus-mediated gene transfer for cystic finbrosis: Part A. Safety of dose and repeat administration in the nasal epithelium; Part B. Clinical efficacy in the maxillary sinus"	10,11
Y	see the whole document	

X	WO,A,94 12649 (GENZYME CORP) 9 June 1994 cited in the application see page 50, line 22 - line 29 see examples 13,14	12,13 10-13

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 1996	25.07.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Hornig, H

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/03818

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. CELL. BIOCHEM. SUPPL. 18A, 4 - 23 January 1994, WILEY LISS, NEW YORK, US, page 222 XP002007820 D. ARMENTANO ET AL.: "Second generation adenovirus vectors for cystic fibrosis gene therapy"	10
Y	abstract no. DZ 102 see page 222	12,13
Y	NUCLEIC ACIDS RESEARCH, vol. 20, no. 9, 11 May 1992, IRL PRESS LIMITED,OXFORD,ENGLAND, pages 2233-2239, XP002007821 G.W.G. WILKINSON AND A. AKRIGG: "Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector" see the whole document	12
Y	HUMAN GENE THERAPY, vol. 5, no. 10, October 1994, MARY ANN LIEBERT, INC. PUBLISHERS, NEW YORK, US, pages 1217-1229, XP002007822 J.F. ENGELHARDT ET AL.: "Prolonged transgene expression in cotton rat lung with recombinant adenoviruses defective in E2a" see page 1221, right-hand column, line 32 - line 36	12
Y	BLOOD, vol. 84, no. 9, 1 November 1994, SAUNDERS, DULUTH, NEW YORK , US, pages 2946-2953, XP002007823 Y. SETOGUCHI ET AL.: "Stimulation of erythropoiesis by in vivo gene therapy: Physiologic consequences of transfer of the human erythrpoietin gene to experimental animals using adenovirus vector" see the whole document	13
Y	CELL, vol. 68, no. 1, 10 January 1992, CELL PRESS,CAMBRIDGE,MA,US;, pages 143-155, XP002007824 M.A. ROSENFELD ET AL.: "In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium" see the whole document	13

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 96/03818

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	KEYSTONE SYMPOSIUM ON GENE THERAPY AND MOLECULAR MEDICINE, STEAMBOAT SPRINGS, COLORADO, USA, MARCH 26-APRIL 1, 1995. JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT 0 (21A). 1995. 415. ISSN: 0733-1959, 31 March 1995, XP002007825 WADSWORTH S C ET AL: "Regulation of viral and therapeutic gene expression in adenovirus vectors." abstract no. C6-450 see abstract	10-13
P,X	HUM. GENE THER. (1995), 6(12), 1575-86 CODEN: HGTHE3;ISSN: 1043-0342, December 1995, XP000575816 KROUGLIAK, VALERI ET AL: "Development of cell lines capable of complementing E1, E4, and protein IX defective adenovirus type 5 mutants" see the whole document	8
P,X	WO,A,95 11984 (CANJI INC) 4 May 1995 see the whole document	8
P,X	HUM. GENE THER. (1995), 6(10), 1343-53 CODEN: HGTHE3;ISSN: 1043-0342, October 1995, XP000575818 ARMENTANO, DONNA ET AL: "Characterization of an adenovirus gene transfer vector containing an E4 deletion" see the whole document	10,11
P,X	GENE THER. (1996), 3(5), 458-465 CODEN: GETHEC;ISSN: 0969-7128, 1996, XP000575875 ZABNER, J. ET AL: "Adenovirus -mediated generation of cAMP-stimulated Cl- transport in cystic fibrosis airway epithelia in vitro: effect of promoter and administration method" see the whole document	10-12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

'ernational application No.
PCT/US96/03818

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 6 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justufying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter vnal Application No
PCT/US 96/03818

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9412649	09-06-94	AU-B-	5734994	22-06-94
		CA-A-	2145641	09-06-94
		EP-A-	0673431	27-09-95
		JP-T-	8503855	30-04-96
WO-A-9511984	04-05-95	AU-B-	8125094	22-05-95
		CA-A-	2173975	04-05-95
		AU-B-	2637295	18-12-95
		WO-A-	9532020	30-11-95